

# Researches on **CELLULAR SLIME MOULDS**


**Selected Papers of  
J.T. BONNER**





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# **Researches on CELLULAR SLIME MOULDS**

Selected Papers of J. T. Bonner



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## Acknowledgements

Prof. J. T. BONNER visited India in the winter of 1990–1991 under the auspices of the Indian Academy of Sciences as RAMAN PROFESSOR. To commemorate this visit the Academy decided to bring out a volume of his Selected Papers. John Bonner readily agreed to this suggestion, and we are most grateful to him for this.

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G. Srinivasan  
Editor of Publications  
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## Foreword

If there is one person who, more than anyone else, has been responsible for the recognition that the cellular slime moulds—in particular, their most widely studied species, *Dictyostelium discoideum*—constitute an ideal system for looking at many of the problems of developmental biology, it is Professor John Bonner. In the course of sustained work carried out over nearly 50 years, he has shown an amazing ability to pick the “right” questions, design deceptively simple but incisive experiments, and, not the least, to present his findings in articles which are models of scientific writing at its best; this attention to style in writing, to a lightness of touch and clarity, comes through even more forcefully in his books. It is a measure of the role played by John Bonner in establishing the field that many of us keep asking the same questions he asked first – and, often, getting the answers he did as well, though that does not always inhibit us from publishing our work! In this Foreword we can mention just a few of John Bonner's major contributions to the field of slime mould biology: the crucial finding that chemotaxis is responsible for aggregation; the elegant observations on photo- and thermotaxis in the slug; the demonstration that pre-aggregation differences foretell the eventual fate of a cell; the pinning down, with Konijn, Barkley and Chang, of cyclic AMP as the long-sought acrasin; the finding that cyclic AMP could also direct differentiation, and, in publications spanning over forty years, the demonstration that ammonia is the “spacing substance”.

The present collection speaks for itself as a survey of his scientific publications; but for those who are not acquainted with them, it may be of use to say a few words about his books. At last count there were ten of them, and it is interesting to see the themes they share in common. Two that strike one straightaway are the idea that developmental patterns are the result of evolution – what evolves is a life cycle as a whole, not a phenotype in “bits and pieces” – and the idea that growth and form must have been of prime importance in the evolution of differentiation. Motivated perhaps by his own experiments on social amoebae, John Bonner has gone on in his books to examine, from the viewpoint of an evolutionary biologist, more complex social interactions leading all the way to the evolution of culture. In the light of the suggestions one hears increasingly these days in favour of a pluralistic attitude to evolutionary theory, it is noteworthy that he remains a more or less uncompromising selectionist.

The impact of his books can be gauged from the fact that at last count, *The Ideas of Biology* had been translated into eight languages and *The Evolution of Culture in Animals* into six. His influence on students of evolution and development, especially beginning students, has also been felt through his popular writings in *Scientific American*, where he is their most published author. It may not be widely known that he played the lead in the highly successful BBC TV film, “Professor Bonner and the Slime Moulds”. Some time ago, the statement that one of us worked on slime moulds elicited the quick and pleasantly surprising response, “The same as Professor Bonner? I have seen him on TV”.

John Bonner was born on 12 May 1920 and had his early education in Harvard University, going back there to complete his Ph.D. after a stint in the U.S. Air Corps from 1942 to 1946. He joined Princeton University in 1947 and has remained there ever since, continuing his researches after formally retiring in 1990. Several honours have come his way, including Membership in the U.S. National Academy of Sciences, and he has served on the Editorial Boards of many journals.



As those of us who have had the pleasure of either working with John Bonner or seeing him in action at close quarters know, in spite of all his success he has remained a modest person, one who values highly a friendly relationship with coworkers and colleagues. He has a positive attitude to life and science, and he transfers this to anyone who comes in contact with him. While in Princeton as a post-doctoral fellow, one of us had a desk in the anteroom to his office. Because he was Chairman of the Biology Department at that time, his fellow professors came to him with all sorts of problems. The question was usually "John, do you have five minutes?" Even knowing that five minutes was the unrealistic minimum, he always had time and took the petitioner into his office. One could not make out what they said to each other, but usually laughter was heard. Although John may not have solved the visitor's problem, he often helped him to live with it. This probably remains the most enduring impression that one has of John Bonner: that he is a man at peace with himself, and that he has both the capacity and interest to help others when help is needed.

The present collection is a must, not just for cellular slime mould fans, which automatically means John Bonner fans, but also for everyone interested in getting acquainted with the central questions which have guided research on this remarkable developmental system. Happy reading!

Edward Cox  
Vidyanand Nanjundiah  
Bernd Wurster

## Introduction

I first became interested in cellular slime molds when I was an undergraduate at Harvard University in 1940. Beginning in my freshman year I fell under the spell of Professor William H. Weston. He was a cryptogamic botanist and a student of his predecessor, the illustrious Roland Thaxter. 'Cap' Weston was a man of exceptional warmth topped with a wonderful sense of humor. Furthermore, he had a great enthusiasm for experimental studies on lower plants; an enthusiasm that was contagious and I caught the disease. At the same I felt that the subject with the most interesting problems for the future was developmental biology, which then consisted primarily of animal embryology. (In fact the term "developmental biology" was not invented until some years later by Paul Weiss). How could I reconcile these two great passions of my biological youth? The answer, I felt, was to find some lower organism that could be used to study development.

At first I was greatly tempted to use water molds (phycomycetes) as an experimental organism, but by chance I found in Cap Weston's outer office a copy of Kenneth Raper's Ph.D. thesis which he had done under Weston a few years earlier. I became enormously excited and wrote immediately to Raper, asking him for reprints of his early work and for a culture of *Dictyostelium discoideum*, his newly discovered species which has become so central in all the many studies on slime molds. On the top reprint he wrote "with the hope for your continued interest in these organisms". In later years we became good friends and I used to twit him that perhaps he now regretted his early wish.

It is surprising to me to think that during the next few years he and I were the only two people working on the cellular slime molds. I can remember during that period, when I gave seminars on my experimental research, the organisms were so unfamiliar to biologists in general that I had difficulty getting beyond a description of the life cycle and on to my experiments. The wonders of the life cycle were quite sufficient to enthrall the audience. The fact that growth occurred first in separate, independent amoebae which, after finishing off the supply of bacterial food, then came together by aggregation to form a well organized multicellular organism was so different from the way all familiar animals and plants developed that my audience seemed not to be able to concentrate on my experiments. After a seminar I gave at the Marine Biological Laboratory in Woods Hole I received a letter from the science reporter at the *New York Herald Tribune* saying he understood I had done something more important than inventing the atomic bomb; I had created a multicellular organism. I assured him that God had done that and fortunately for me the reporter restrained his journalistic zeal. Jumping from this low point in my early career to a high point, I was asked to come to Yale to give a seminar when I was still a graduate student. After the seminar, which was attended by what seemed a paralyzingly large number of people, Professor Ross G. Harrison, then in his 80's whom I, along with everybody else, considered to be the world's greatest living embryologist, came up to me and said if he were starting all over again, he would work with slime molds. It was a moment for me that made walking on clouds a simple matter and I can feel the glow to this day.

The early work by both Raper and me, and indeed our later work as well, was primarily biological. I have often asked myself whether or not this was a wise course; would it not have been better if I had followed the new trends by working on the biochemistry of development and, more recently, on the molecular biology of development? I will never know the answer to this question, but I do believe that there is great merit in pursuing development from all three

directions, and that the experimental biologist often leads the way by delineating the nature of the problem. He or she is in a better position to see the whole picture and frame the questions that need to be answered.

In the early 1950's there was an influx of new workers who concentrated on the biochemistry of slime mold development. The main credit for this wave of activity should go to Maurice Sussman, not only for his own work, but for the large number of bright graduate students and postdoctoral fellows who worked with him. It was generally thought that cellular slime molds and *Dictyostelium discoideum* in particular provided the ideal opportunity to study eukaryotic development in the way the development of *E. coli* had been so successfully analyzed. The next big advance of the biochemical attack started in Europe, stimulated by the work of Günther Gerisch in Germany. He and his collaborators made a series of exciting new discoveries on the biochemistry of adhesion, especially during the aggregation phase, which did much to explain many of the intriguing biological observations Brian Shaffer had made earlier on cell adhesion changes during development.

This movement in the biochemistry of development spread rapidly and spawned a number of important centers of activity, such as Peter Newell and John Ashworth in Britain, Theo Konijn in the Netherlands, and Ikuo Takeuchi in Japan. This is certainly not a complete list and I would be remiss not to mention James Gregg, the pioneer who did the first work on the biochemistry of development. There are many others who have made important contributions that, for want of space, I have not listed and to whom I apologize.

While this biochemical wave spread, the number of workers in the field made a second megajump with the advent of molecular biology. There the first adventurers were Harvey Lodish and Richard Firtel who began to take seriously the possibility that cellular slime molds were most suited to yield to the new molecular techniques. Today there are a large number of molecular biologists working with slime molds and they have made and are making significant advances in our understanding of the genetic control of development.

It is interesting to note that for a long time the stumbling block was genetics and it was for this reason that work on yeast, *Drosophila* and nematode development has sailed ahead of the slime molds. Initially the problem was that the life cycle of cellular slime molds is asexual, and the chromosome number haploid. There was no known sexual cycle. This was partially solved by Maurice Sussman and his co-workers who discovered that like *Aspergillus* there was a parasexual system and that it was possible to make crosses and get progeny through mitotic recombination of the separate amoebae prior to aggregation. This was followed by William Loomis showing how to make diploids with temperature sensitive mutants, a discovery that was successfully exploited by a number of workers besides Loomis, especially Keith Williams, Peter Newell, Jane Warren and Edward Cox.

Great progress was made with these methods, but they fell far short of what one could do if one had true sexuality. Macrocysts were first discovered by Kenneth Raper and his group, and, along with Michael Filosa (in collaboration with a colleague skilled in electron microscopy) more and more evidence was accumulated to indicate that the macrocysts were zygotes and were the main stage of a sexual cycle. The final coup came when David Francis and his co-workers, as well as Raper's group, demonstrated mating types among different strains of the same species. Unfortunately there is one remaining difficulty in using this sexual stage for genetic studies. The barrier is in germinating the macrocysts; the percentage of success in any one experiment is too low to be of practical value. Hopefully the day will come when this last difficulty will be removed.

However, through the powerful new methods of molecular biology it is now possible to



partially circumvent the need for conventional genetic methods. There has been a new and important surge of work on slime molds in which it is now possible to isolate, clone and identify the structure of specific genes and their proteins and to examine their role in development. The new methods have put slime molds almost on par with *Drosophila* and *Caenorhabditis*; now it is important to catch up with them. The main reason for this is that all three groups of organisms develop in such different ways that the comparative study of their respective molecular genetics of development is bound to be wonderfully rewarding.

As will be seen from the papers that follow in this volume, my own interests and approaches have remained with one foot firmly planted in the biology of slime molds. Where biochemical matters have come into my research it has always been with gifted collaborators who showed me the way. Initially my interest was in the mechanism of aggregation and in my doctoral dissertation I provided evidence that chemotaxis was involved. This seems fairly obvious to us today, but at that time it had to be clearly demonstrated to convince other biologists. The attractant, which initially I called *acrasin*, was identified by Theo Konijn, a visitor from Holland and David Barkley, then a graduate student. The idea was based on work Konijn had done in the Netherlands. I was not even in the laboratory at the time; they called me in Canada to tell me that their inspiration worked: cyclic AMP attracted amoebae in wonderfully low concentrations. Later Pauline Pan, a research associate in our laboratory, showed that folic acid was also an attractant. In the case of the acrasin of *Polysphondylium* it was first Bernd Wurster, a postdoctoral fellow, and then Osamu Shimomura, then from another laboratory at Princeton, who finally showed that *Polysphondylium* acrasin was a curious dipeptide which we named *glorin*.

Biological experiments on development have also been important in pointing the way for the study of differentiation. Why do some cells become spores and others stalk cells, and how are their proportions controlled? The answers to these questions will be molecular, but again the framing of the question in the first place has invariably been from straightforward biological observations and experiments.

There is another approach to development that has considerable potential and already has shown itself to be useful and even powerful. It is the use of mathematical modelling. This is already evident from the work of Harry MacWilliams and Vidya Nanjundiah on the prespore-prestalk proportioning in *D. discoideum* and the work of Edward Cox and his collaborators on the beautiful symmetry and geometry of the fruiting bodies of *Polysphondylium*. Such models can lead to reasonable hypotheses on how the pattern is achieved, and those hypotheses can then be tested experimentally. Modelling can sometimes suggest new solutions that otherwise would not have been even imagined.

There is a fine line between experiments that bear on physiology rather than development. There is the whole problem of how the migrating slug moves, and how it orients with such incredible sensitivity to directional light, heat gradients and gradients of a volatile product of metabolism, which turns out to be ammonia. Again the initial observations were simple biological ones, but when one wants to find out what the mechanisms are behind these behaviors one must look for biochemical and biophysical mechanisms that govern these reactions to the micro-graded properties of the immediate environment of the moving cell mass.

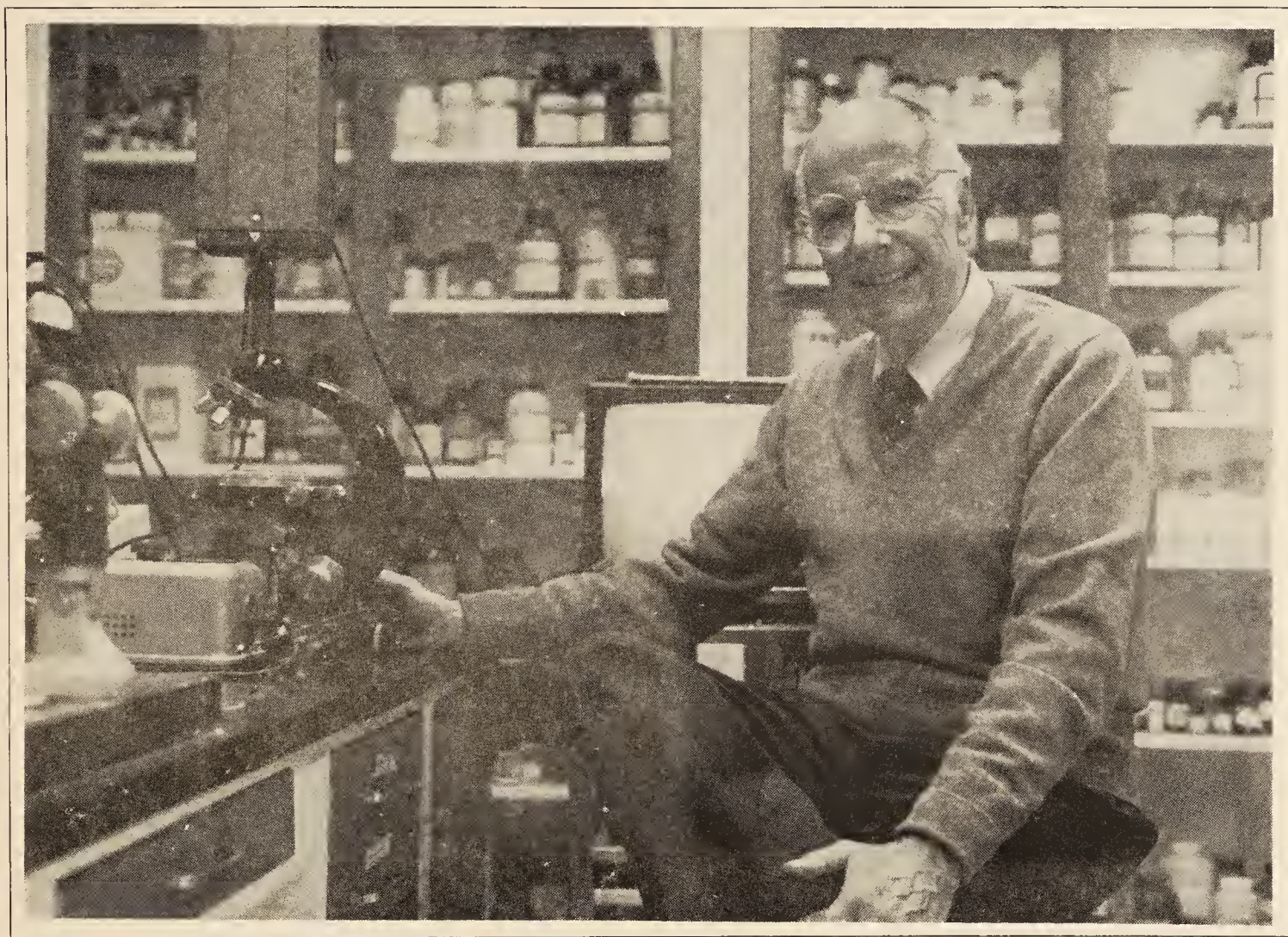
Besides physiology there has been considerable interest in the evolution and ecology of the slime molds and many have made important contributions including James Cavender who has done the most to understand the world-wide distribution of cellular slime molds. I have been especially absorbed by the question of their evolution, a matter of great interest and importance.

It is often said (especially in the writing of research proposals for grants, a process that

tends to govern our lives) that cellular slime molds are a good model system. By this one presumably means that one can apply lessons learned using slime molds to our understanding of vertebrate development, and especially human development. To me this way of thinking has always been deeply troublesome. My fascination with the experimental analysis of slime molds is based on the idea that one wants to find out about slime molds! Slime molds are a model system for the study of slime molds. If one reveals some activity or process that is shared with other organisms, then all to the good; but that is not the reason I have devoted my scientific life to slime molds. Rather, it was to see how slime molds work, especially in their development, and then to compare them to mammals and other vertebrates, to invertebrates such as nematodes and fruit flies, to fungi such as yeast, and to higher plants. The fact that each of them will have important differences and important similarities is to me the key issue. It is only by making a comparison of such diverse developments that we will have a genuine understanding of the diversity and the unity of life.

**John Tyler Bonner**





Professor J. T. Bonner at his laboratory in Princeton.





## A DESCRIPTIVE STUDY OF THE DEVELOPMENT OF THE SLIME MOLD *Dictyostelium discoideum*<sup>1</sup>

John T. Bonner

**THE ORGANISM AND ITS IMPORTANCE.**—The slime mold *Dictyostelium discoideum* is a member of the Acrasiales. The historical background of the Acrasiales is most adequately treated by Olive (1902). He emphasizes the pioneer work of Brefeld (1869, 1884), Cienkowski (1873), and van Tieghem (1880), but the first and, to date, the only really comprehensive treatment of the group is that of Olive himself.

He pointed out that the Acrasiales are a group of the slime molds which are distinguished principally by the fact that throughout their life cycles they are made up of uninucleate, amoeboid units (with the exception of *Sappinia*, which is binucleate), never becoming multinucleate as do the true myxomycetes. They differ further from that group in lacking sexuality (Skupienski, 1918, is the only investigator that has taken exception to this statement, but his view is generally not accepted), and nowhere in their cycle do they possess flagella as a means of locomotion. The order includes three families: the Sappiniaceae which produce extremely simple fruiting bodies of one or several amoebae; the Guttulinaceae which, although they incorporate a large number of cells in their fruiting body, are irregular and not highly differentiated; and, finally, the Dictyosteliaceae which are composed of very complex structures, unbranched (*Dictyostelium*) and branched (*Polysphondylium*).

Raper's (1935) discovery of *Dictyostelium discoideum* was a significant advance, because that particular organism was better suited for experimental work than any other of the Acrasiales. The following description of the life cycle (fig. 1, above) is extracted from his excellent papers (Raper, 1935, 1937, 1939, 1940a, 1940b, 1941):

The spore germinates under suitable circumstances, liberating a myxamoeba which, by binary fission, will produce many of its own kind. This is then a period of growth and multiplication for which food is essential; consequently, it is called the *vegetative stage*. The myxamoebae feed on bacteria of many kinds in the ordinary phagocytic manner; in fact this is the only way that they can assimilate food, at least so far as is known (Raper, 1937). The general shape and distribution of the myxamoebae will depend almost entirely on the shape of the bacterial colony. If the bacteria are distributed evenly

over the agar surface the myxamoebae will be also, like stippling on a drawing board.

With the consumption of most of the bacteria, the vegetative stage stops, and the *aggregation stage* sets in. From this point on, all growth and vegetation ceases, and morphogenesis alone begins. The myxamoebae which previously had no relation to each other suddenly have a very definite one, all becoming oriented towards a central point and aggregating towards it. As aggregation proceeds the myxamoebae group together more and more, giving the appearance of little viscous streams flowing into a central mound. The mound rises to a "slug" or cartridge-shaped *migrating pseudoplasmodium* which then falls to its side and migrates over the substratum for a variable length of time. When this ceases the "slug" again rights itself to start what has previously been called the "sorocarp formation" stage, but it seems advisable to use the term *culmination stage*, both for the sake of euphony and more exact meaning. In the course of culmination the *sorus* (or spore mass) is raised into the air, attached to the top of a delicate *stalk* (or sorophore), which in turn is attached to a round basal disc. During this process, the whole of the rising mass (everything other than the visible portion of the stalk and the basal disc) is called the *sorogen*, a convenient word suggested by Harper (1926, 1929).

This cursory picture of *Dictyostelium discoideum* that has been extracted from the literature is sufficient to illustrate just why it might be a useful organism in experimental studies on morphogenesis. It not only has advantages common to all members of the Acrasiales, but ones which no other representative of the group possesses. (1) It can be cultured easily in the laboratory under room conditions; (2) its normal nutritional requirements are well known (Raper, 1937, 1939); (3) since the environment is that of a culture dish it can be controlled; (4) the life cycle is short, lasting only four days; (5) perhaps the most important point is that the morphogenesis is separated naturally from growth in the development of the organism. This is an ideal situation for those interested in biological form, for here is a natural separation of the two form-producing factors which are ordinarily so closely connected that it is impossible to determine the roles of each. (6) There are only three types of cells (stalk, spore, and basal disc) and these have a similar parent cell; (7) depending on the number of amoebae in one pseudoplasmodium (varied by natural causes or operation) proportional fruiting bodies of a great size range can be obtained; (8) because the pseudoplasmodium is naked it can be operated upon easily, making grafts, etc. (especially true of the migration stage).

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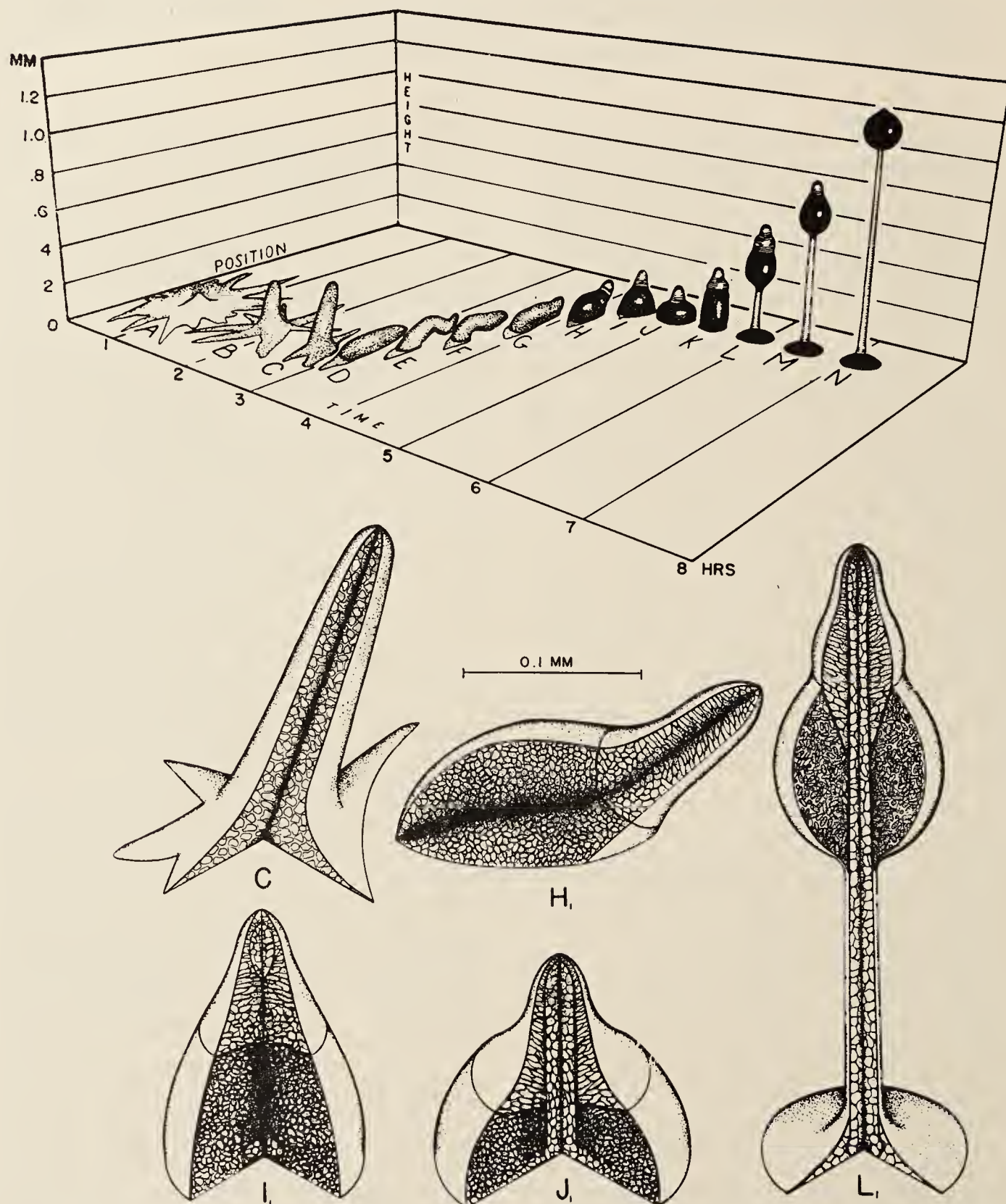


Fig. 1. (Above) The complete morphogenesis of *Dictyostelium discoideum* is represented in a three dimensional graph. A-C, aggregation; D-H, migration; I-N, culmination. The presence of pre-spore cells is indicated by heavy stippling, H-K; and the presence of true spore cells by solid black, L-N. (Below) Semi-diagrammatic drawings of the external and internal appearance at five different stages. C<sub>1</sub>, end of aggregation; H<sub>1</sub>, end of migration, showing the presence of pre-spore cells; I<sub>1</sub>, the beginning of both culmination and stalk formation; J<sub>1</sub>, the flattened stage of culmination with the completion of the lower portion of the stalk; L<sub>1</sub>, culm-2 with active stalk formation at the tip, with newly acquired true spore cells, and with a completed basal disc.

PURPOSE.—The purpose of this paper is twofold. One is to add to the external descriptive knowledge of the organism. The other is to understand the

cellular structure of the organism, and especially to further a study that Raper (1940b) has already initiated: that of tracing throughout the develop-



ment of the organism the course of the position of any particular cell or group of cells in relation to the whole organism.

**MATERIAL AND METHODS.**—The development of the organism was considered from two different points of view: (1) the external appearance, and (2) the cellular structure.

1. The most successful method of getting a complete picture of the external shape was to take photographs at fifteen-minute intervals, each picture being placed along side the others in a series to give "successive pictures" of the changes in shape (figure 2 is an example of this method). These photographs were obtained in the following manner. A block of agar with the organism located on top of it was cut out from a petri dish, put in a van Tieghem cell and sealed in with a coverslip smeared with vaseline. To prevent condensation a glass tube was wrapped three quarters around the van Tieghem cell, and a slow current of cold water from the faucet was allowed to pass through it. The light source was from a simple microscope lamp, the beam of which passed through a water cell to absorb the heat, and a magnifying lens to converge the light upon the side of the developing organism. The mounted van Tieghem cell was placed in a horizontal microscope which had a 35 mm. miniature camera attached to the ocular end. The winding spool of the camera was geared directly to a small electric motor, the function of which was to turn the film between exposures. The camera was always left open and the exposures were made simply by turning on the microscope lamp. The time and the extent of the exposure was controlled by a Navez timer. (Dr. Navez is preparing a description of this timer that should appear shortly in *Science*).

The movement of the pseudoplasmodium in fruiting was best brought out by time-lapse motion pictures. The apparatus was a Bausch and Lomb ciné-photomicrographic outfit attached to a horizontal microscope, which held the van Tieghem cell in much the same manner as described above for the "successive picture" method. The time lapse used was one frame every thirty-two seconds or, in other words, one minute on the screen is equivalent to eight and one half hours of actual development.

When the movement was to be recorded over short intervals of time, which was especially desirable in the case of the migrating pseudoplasmodium, camera lucida drawings at known intervals were very useful. This was done most easily in a petri dish under the low power of the compound microscope.

2. Among the techniques for examining the cellular structure, the vital staining of the aggregation streams with neutral red proved helpful for following particular amoebae for short lengths of time. A fairly fine capillary tube was attached to a crude Barber micromanipulator. Under the dissecting microscope a small drop of vital stain was blown from the end of the capillary upon the aggregating stream.

Paraffin sections of the organism at various stages of development proved invaluable in determining the

internal structure. Carnoy's fixative was used, but more successfully a solution containing 3 parts saturated  $\text{HgCl}_2$ , 2 parts glacial acetic acid, and 5 parts 95 per cent alcohol, followed by a wash of  $\text{I}_2$  in 70 per cent alcohol (a rapid fixative is necessary to coagulate the loose pseudoplasmodium quickly). Ten cubic centimeters of fixative were poured gently into a petri dish containing organisms at the right stage of development. After a few minutes the pseudoplasmodia were sucked up with a pipette and squirted gently into half of a shell vial with the lip end covered with a cloth net. In this way one can concentrate all the fruiting bodies, perhaps a hundred, in the tip of the sieve-like vial. Then, by merely dipping this vial in the various solutions one can proceed through the alcohols, cedar oil, xylol, to paraffin, stopping in 95 per cent alcohol with eosin so that the tissue can be seen. The contents of the vial were poured out while in the paraffin and carefully massed together in a heap, ready for solidifying. The sectioning was done at  $5\ \mu$ . Harris's haematoxylin was the most successful stain used.

So many pseudoplasmodia in one paraffin block, according to the laws of chance, will yield some at the right stage and cut at the right angle. A few especially interesting serial sections were drawn with a camera lucida. Then each section was traced upon a separate glass plate with a brush and india ink. By means of a clamp the plates were held the right distance from one another, giving a simultaneous picture of the internal and external structure. Total mounts were also valuable and were made by the same staining and fixing methods as those mentioned above.

**EXTERNAL APPEARANCE.**—All the basic descriptive information on *Dictyostelium discoideum* will be found in the various papers of Raper (1935, 1940a, 1940b). In this study a few additional facts on the external appearance were revealed by the different techniques that were employed.

**Aggregation.**—The aggregation of *Dictyostelium discoideum* is very similar to that of *Dictyostelium mucoroides*, the most complete descriptive information of aggregation being that of Arndt (1937) on the latter form. Arndt took time-lapse motion pictures of this stage and described the results obtained by this technique in considerable detail. Perhaps it is well to mention one of his points here, for it is similar to the activities found in later stages. He found that the incoming streams have centrifugal pulsations which radiate from the center to the extremities of the streams.

In the present work on *Dictyostelium discoideum* an interesting observation was made. When aggregation is finished it has produced the slug-shaped migrating pseudoplasmodium. Throughout aggregation, therefore, the whole development of the pseudoplasmodium is leading up to that structure. It gradually rises from the agar surface into the air until the aggregation streams have completely entered into it. Then the vertical projection will suddenly topple over and lie flat along the agar surface. The



shapes of the steps described here and drawn in figure 1 represent a typical, normal individual. If the conditions are slightly adverse (i.e., subjected to a dry atmosphere for a period), one can completely eliminate the migration stage. In this case the aggregation morphology is quite different. Right from the beginning the amoebae pile up a structure that resembles a pseudoplasmodium at the onset of culmination (fig. 1, I), just as in normal aggregation the structure resembles a pseudoplasmodium at the onset of migration. The important principle to be derived from these two facts is that the aggregating pseudoplasmodium has no real constant shape of its own, but prepares the amoebae for the shape they are to assume as soon as migration is over.

It should be mentioned that the movement involved in aggregation is a *morphogenetic movement*, for the general shape of the pseudoplasmodium is undergoing change, and, therefore, inevitably, the cells are moving in relation to each other and the structure as a whole.

*Migration.*—From the time-lapse motion pictures (seen especially well in a short strip of film taken by

K. B. Raper) the fact was brought out that the large portion of the migrating pseudoplasmodium moves smoothly, without any jerking, while the tip will occasionally pulsate slightly.

It was also found, by means of the camera lucida drawings, that the various parts of the pseudoplasmodium behave differently. In the first place the posterior end probably moves at a constant speed for quite extended periods of time. The speed of individuals may vary from about 0.5 mm. to 2 mm. per hour. The tip, however, is very irregular in its movements, and this irregularity is believed to correspond with the slight, uneven pulsations seen in the motion pictures. Furthermore the cause of this irregularity seems to be quite obvious. If the tip is touching the agar it progresses slowly, and becomes broad, but if it is in the air it becomes narrow and lunges forward. It resembles the human tongue in its ability to assume different shapes (fig. 1, D, E, F, G, H).

There is some variation in the shape of the migrating pseudoplasmodium as pointed out in the above paragraph, but it always maintains approxi-

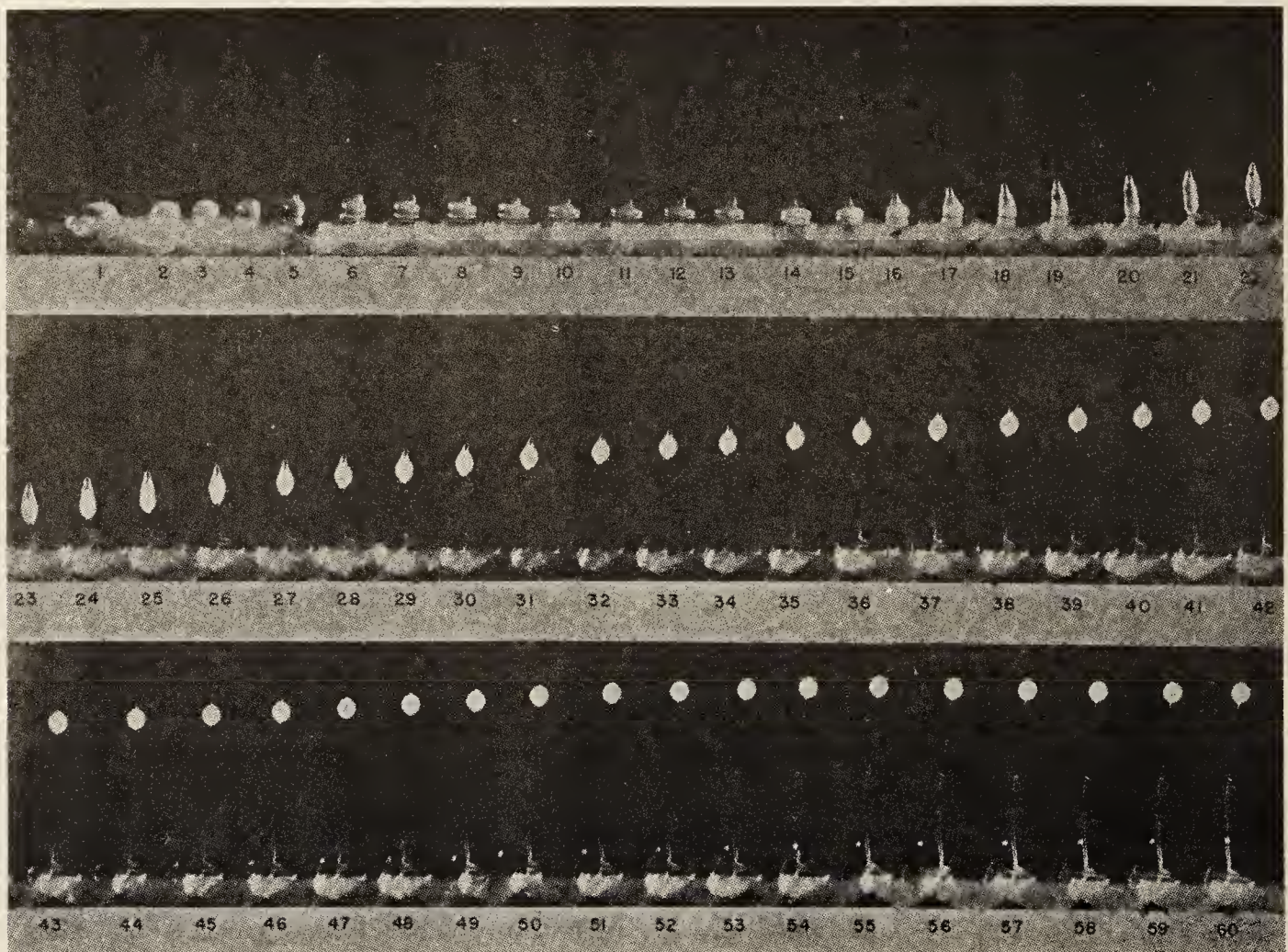


Fig. 2. Culmination of *Dictyostelium discoideum* shown by a series of photographs taken at 15-minute intervals. 1-4, the migrating pseudoplasmodium; 5, the pseudoplasmodium begins culmination; 6-14, flattening of the culminating pseudoplasmodium; 20, culm-1; 26, culm-2; 30, culm-3; 35, culm-4; 41, culm-5; 44, culm-6; 47, culm-7; 50, culm-8. The mature sorocarp, 55-60, culm-8.5, is about 2 mm. high. In 21-24 note the formation of true spores, which is indicated by the sorogen becoming opaque. Also note that in the bottom row a smaller sorocarp has developed.



mately the shape of a cartridge (fig. 1, D, E, F, G). It is only just prior to culmination that it assumes a more rounded shape (fig. 1, H).

Yet these variations in contour are very slight, and therefore, despite the fact that the migrating pseudoplasmodium moves as a whole, it is not a morphogenetic movement. This will be further verified later in the examination of the cellular structure.

**Culmination.**—It was observed in the time-lapse motion pictures that in the transition from migration to culmination, when the long axis of the pseudoplasmodium is raised to a vertical position, this does not occur by pushing upward in a straight line, but by curious undulating, snake-like movements. However, this only occurs when the tip is very long, for if the tip is short, as in figure 2, 5, the process is uneventful and smooth.

Once the pseudoplasmodium has reached the upright position (fig. 1, I, I<sub>1</sub>, fig. 2, 5) that marks the beginning of culmination, an important event, not previously mentioned in the literature, occurs. The total height of the pseudoplasmodium becomes visibly reduced as its diameter becomes visibly larger, assuming almost the shape of an automobile tire (fig. 1, J, J<sub>1</sub>, fig. 2, 6-14). The extent of this flattening is extremely variable, but no instance has yet been seen where it could not be observed to some extent. Following this decrease in height there is a corresponding increase in height that continues till the spore mass is high in the air and has reached its maturity. This process is smooth and gliding, except in the case of the peg-like tip that protrudes above the sorus in the rising sorogen, which pulsates and rises by jerks. This phenomenon is particularly marked in the earlier stages of the final elevation.

It is timely to mention here a convenient and exact system of designating the degree of advancement of a culminating pseudoplasmodium. *Culm-1* is considered the stage where the sorogen is on the very verge of leaving the substratum. It is called 1 because the stalk height =  $1 \times$  sorogen height. At *culm-2* the stalk height =  $2 \times$  sorogen height. This relation continues to about *culm-7* or -8, which is the usual, normal proportion between stalk and sorogen for the mature sorocarp, but if conditions are not ideal, development may cease at a very much earlier stage (Raper, 1939). If the pseudoplasmodium under observation is intermediate between two such stages, its accurate numerical value is given (for example: *culm-3.5*, because stalk =  $3.5 \times$  sorogen).

In closing this section it should be pointed out that the movements that take place during culmination obviously involve changes in shape and therefore they are solely morphogenetic movements, as they were in aggregation. Migration, the stage that can be eliminated under special conditions is coincidentally the only stage whose movements are not morphogenetic in nature.

**CELLULAR STRUCTURE.**—There is no record in the literature of any attempt systematically to use histological fixing, staining, and sectioning techniques

to reveal the internal structure of any of the Acrasiales. It was found that through these techniques a great deal of new information could be obtained about *Dictyostelium discoideum*.

**Aggregation.**—Since the incoming streams of the aggregating cells are very thin, commonly only one amoeba thick, they have been observed by all the investigators, and the elongate shape of the amoebae, the clear portion at their anterior, or better, centripetal end, and their coming-together in streams have been thoroughly described and illustrated.

What has not been adequately described is the cellular structure of the heap of central cells that the aggregation streams build up. In an attempt to attack this point, it was found that the best picture of these was obtained from carefully fixed and stained total mounts. The cells appear to be quite similar to those of the incoming streams except that they are not so elongate. They are large and irregular in shape and not at all highly compacted, that is, the spaces between the cells are distinctly visible (fig. 1, C<sub>1</sub>).

**Migration.**—It is difficult to get a very clear picture of the cellular structure of a young migrating pseudoplasmodium. It differs from a late aggregating pseudoplasmodium in that the cells are smaller and more compacted. This is not true of the large and loose cells at the very posterior end which drop behind in the slime sheath. The fact that they are larger may merely be caused by the fact that they are out of the pseudoplasmodium body, for if a pseudoplasmodium body is crushed with a needle and the cells watched under a microscope, in less than a minute they can be seen to swell considerably and assume approximately the same size as the vegetative cells. This is perhaps the best way to see how much smaller the fruiting cells are than the vegetative cells.

Often a dorso-ventral orientation will be seen in the cells of the apical third, but this is not necessarily true. The posterior two-thirds, or if there is no dorso-ventral orientation, the whole pseudoplasmodium, is made up of fairly rounded cells, homogeneous in their shape and structure, showing no orientation.

Toward the end of migration these characteristics become much more accentuated (fig. 1, H<sub>1</sub>). The cells of the posterior third become even rounder and more compacted, in fact, they appear now quite distinct from the apical cells, the division line being very sharp. This distinction, when the cells are stained with Harris's haematoxylin, becomes very clear, for the whole posterior cell mass stains much more heavily. Their dark staining, their compactness, and sharp boundary line separating them from the apical region all indicate that they are to become spore cells. These cells, first found near the end of migration, will be called *pre-spore* cells. The cells of the now more elongate portion are lighter in their staining reaction, less compacted, and nearer the tip. They are conspicuously oriented in a dorso-ventral direction.



*Culmination.*—When the pseudoplasmodium rights itself at the beginning of culmination, the anterior cells that had a dorso-ventral orientation now become horizontal. In this anterior portion a group of cells near the tip becomes rounded off and enlarged. They are not yet vacuolated, they take the stain evenly, although more lightly than the surrounding cells. The whole group is either round or oval in shape and its outer limit is smooth, bounded by a visible wall, similar to, but thinner than, the cellulose-like stalk wall of the mature sorocarp (fig. 1, I<sub>1</sub>). There was one instance where this process occurred at the end of migration).

Before following the development of this particular group of cells, there is another somewhat similar group at the base of the pseudoplasmodium. The outline of this group is irregular, and there is no appearance of an outside membrane. The cells themselves are quite advanced in their enlargement; in fact they are almost always found to be already vacuolated. Not only are the outlines of the group irregular, but individual vacuolated cells may be found at some distance from the group, although always in the general vicinity of the central, basal portion. These cells, of course, are entirely surrounded by the pre-spore cells. One of the interesting features about them is that they do not seem to be always present, but only in about half the cases. Whether their time of appearance is variable, or whether their actual presence is optional, is an unsolved question. Their general shape is usually somewhat flattened, the long axis being horizontal rather than vertical. They give the appearance of being the bulbous base of the stalk, but there is also the possibility that they might contribute to the basal disc, although at this stage there is no other sign of a basal disc (fig. 1, I<sub>1</sub>).

As development proceeds and the pseudoplasmodium begins to acquire its flattened position, there is no evidence that anything occurs to the stalk cells in the basal portion, but those in the tip become very active. While the general contour of this upper group of the stalk cells had been spherical or oval, it now assumes the shape of an inverted tear drop. The cells in the most posterior portion of the drop, the narrowest portion, now become vacuolated, and are progressively less so as one proceeds toward the tip. This narrow wedge continues to forge its way downward into the pre-spore cells, till it finally joins the cells at the base, forming a complete stalk (fig. 1, J<sub>1</sub>). All the cells are vacuolated except those at the extreme tip. It is uniformly cylindrical except for the club-like swelling at the extreme tip and the bulbous base. This base is set in the beginning of the basal disc. The disc is not clearly visible at this stage, except for a few swollen vacuolated cells. It first becomes clearly visible when the pre-spore mass leaves it.

It seems evident that the flattening of the pseudoplasmodium during culmination is associated with stalk formation, for this process shortens the gap that the stalk cells must bridge. There is one impor-

tant point that is not satisfactorily established. Are the cells in the lower part of the stalk cell mass pushed downward, or do they arise *in situ*? They give the appearance of having been pushed downward, and if this is the case it would harmonize with the flattening of the whole pseudoplasmodium, during which the earliest formed stalk cells are pushed down through the pre-spore cells to the base.

This view seems even more attractive when one considers the formation of the stalk in the rising sorocarp. The upper cells surrounding the stalk are still oriented in the horizontal manner, although they are now more oblique in respect to the stalk than they were, and they are pierced by the firm-walled stalk (compare fig. 1, I<sub>1</sub>, J<sub>1</sub>, L<sub>1</sub>). The only place where the stalk wall is still thin is at the very apex; in fact at the very top the membrane is so thin that it is even questionable whether it exists, and what one sees is merely a division line between two layers of cells. The sides of the apical region of the stalk bulge out showing signs of flexibility. The few most apical cells above the stalk, and those just below it are very small, dense, and spherical. Inside the stalk, progressing downward, they become first larger, less dense, then finally vacuolated. (Brefeld, 1884; Olive, 1902; Raper, 1940a, have all drawn this stage, but none of their drawings concur exactly with fig. 1, L<sub>1</sub>. The difference can undoubtedly be accounted for by the fact that their illustrations are based on total mounts, while figure 1 is based on serial sections). Now there is only one way in which the cells can enter the stalk and that is over the top of the cellulose-like sheath. If the stalk is added to at the apex, there must be a considerable pressure exerted on the very top of the stalk. If the whole stalk is rigidly fixed to the substratum, this force must be important in raising the sorogen. The structure of the surrounding tissues, the oblique orientation of the cells below the tip, the bulge in the flexible apical portion of the stalk sheath, the small size of the uppermost cells are all probably indications of the existence of such a force.

As the stalk rises, the undifferentiated, obliquely oriented cells in the tip are progressively used up so that they are completely gone in the normal mature fruiting body. The swelling at the tip of the stalk also seems to disappear at the very end.

The fate of the pre-spore cells must also be considered. As the pre-spore mass leaves the substratum it slips away from the basal disc cells which have become vacuolated and closely knit together. Thus far no cellular changes in the pre-spore mass have taken place, but a very rapid change occurs around culm-1 to culm-2. Suddenly, in about thirty minutes, all the pre-spore cells differentiate into true spore cells. This can readily be seen in figure 2, where the opaqueness of the pseudoplasmodium is equivalent to the degree of conversion into true spores. In this particular instance the process begins at culm-1 (this is slightly earlier than average). Figure 2, 20 is completely clear, 21 fairly opaque, 23 almost completely opaque, and 24 completely opaque. The proc-



ess is ended in this case in 45 minutes. Note in 22 and 23 that they are more opaque in the upper region than in the lower. The stained sections explain this, for differentiation starts in the uppermost exterior ring, progresses inward and downward from that region, reaching finally the base of the spore mass. (This is somewhat opposed to Raper's observations, 1940a, but again the difference can be ascribed to the fact that he worked with total mounts, and sectioned material was used here). Sometimes at the base there were a few large undifferentiated cells that appeared almost abnormal and served no apparent function. From culm-2 to maturity the spore mass underwent no further cellular changes.

**DISCUSSION.**—The material covered in the previous sections is not composed of entirely isolated facts, for there is a connecting link that binds all this descriptive morphology. That link is the tracing of the course of the position of any particular cell, or group of cells, in relation to the whole organism, throughout its development. Since there is no cell division after the vegetative stage, there are the same number of cells in the beginning of aggregation as there are at the end of culmination. Each change in external shape is then a direct result of alterations in position of a fixed number of cells. Consequently, if one can follow the course of the individual cells or cell groups, one will have a complete picture of internal and external development.

In respect to the process of aggregation, one must answer the question as to whether or not the incoming aggregating cells have a specific position in the resulting pseudoplasmodium, relative to that which they occupied in the circle cut out by the original aggregation center. A study of this situation reveals that the nearer the amoebae are to the aggregation center, the nearer they will be to the apex, and vice versa. This was ascertained by staining the incoming stream with neutral red and following the stained cells under the dissecting microscope. In the simplest situation, where there was only one large incoming stream, the cells approached the circular central rising pseudoplasmodium and flowed about the outer edge to form a complete outward ring of red. Staining the cells at an earlier stage was not only a more difficult task to perform but also more difficult to observe, yet there was no evidence of the cells piling over one another; the later the cells came in, the more posterior was their position in the pseudoplasmodium (fig. 3).

The next problem is to find out whether or not these cells which have earned their specific position in the migrating pseudoplasmodium keep that position throughout the migration period, or whether the cells mill about during migration and obliterate the original cell pattern. This was answered by an experiment of Raper's (1940b), which showed the former to be the case. By feeding some cultures on the red bacterium *Serratia marscesens*, and others on the colorless bacterium *Escherichia coli*, he obtained both red and white migrating pseudoplasmodia. He then grafted red anterior portions upon

colorless posterior portions and vice versa. In these experiments the line of demarcation between the red and the white, regardless of the length of migration, remained sharp and constant. Therefore, until the end of migration the cell position remained that which was determined at the very onset of fruiting. This also means that the motion of the migrating pseudoplasmodium is performed without morpho-

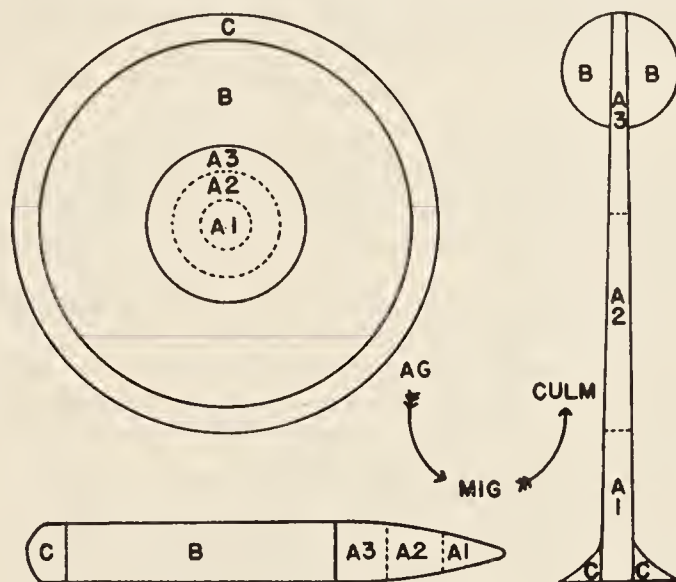


Fig. 3. A diagram showing the position of different cell groups throughout the morphogenesis of *Dictyostelium discoideum*. AG, the aggregation circle; MIG, the migrating pseudoplasmodium; CULM, the mature culminated sorocarp. A, the stalk cells, 1, 2, 3, being arbitrary regions; B, the spore cells; C, the basal disc cells.

genetic movement (i.e., change in the position of the cells).

In the discussion of the cellular structure of the culmination stage it was clear that different cell groups (stalk, spore, and basal disc cells) had already become visibly differentiated. Therefore, it is obvious that the cell positions that were determined at the beginning of aggregation are still maintained at the end of culmination.

An attempt to represent this unified picture diagrammatically is made in figure 3. Like the maps of presumptive areas made by the embryologist, one can see in a diagram of aggregation what the various regions of the aggregation circle are to become. In other words, their fate is determined at the onset of development. (This is not an irrevocable determination, but can be altered by experiment (Raper, 1940b)). Furthermore, one can follow these regions throughout the rest of the development, through migration, through culmination to the final, mature fruiting body. The basal disc cells (fig. 3, C) are peripheral during aggregation, posterior during migration, and basal during culmination. The spore cells (fig. 3, B) are intermediate in the first two stages and apical in the final stage. The stalk cells (fig. 3, A) are central during aggregation, anterior during migration, and lie between the basal disc and the spore mass during culmination. In observing the



different regions of the stalk (fig. 3, A1, A2, A3) one finds that their position becomes reversed from anterior to posterior during culmination. This transformation or inversion of the regions of the stalk has been explained in the discussion of the cellular structure of culmination. The presumptive stalk cells are pushed over the top of the stalk sheath and downward in the direction of the basal disc. This downward push results in the cramming of the stalk cells into the basal region, and, once that is accomplished, it will result in the rising of the sorocarp.

## SUMMARY

The morphogenetic development of *Dictyostelium discoideum* during aggregation, migration, and culmination stages was examined from two points of view: (A) external appearance and (B) cellular structure.

A. From the standpoint of external appearance some new aspects of the development are described:

1. The aggregation-pseudoplasmodium has no consistent shape of its own but prepares the amoebae for the shape they are to assume in the migrating or the culminating pseudoplasmodium.

2. Although the bulk of the migrating pseudoplasmodium moves smoothly, the tip moves irregularly—slowly when it is touching the surface of the substratum, and quickly when it protrudes into the air.

3. During the culmination stage a flattening of the upright pseudoplasmodium takes place before the final elevation.

4. During the early stages of the final elevation of the culminating pseudoplasmodium, the peg-like tip rises by jerks, while the rest of the sorogen rises smoothly.

B. By the use of fixing, staining, and sectioning techniques, a clear picture of the cellular structure was obtained.

1. The cells during migration are smaller and more compact than they are during aggregation.

2. The dense pre-spore cells first become delineated in fairly advanced migrating pseudoplasmodia.

3. The cells in the apical portion of the pseudoplasmodium, from the migration stage until near the end of development, have their long axes at right angles to the long axis of the pseudoplasmodium.

4. The stalk cells arise in the beginning of the culmination period (in one case at the end of migration), first in the center of the apical portion, and soon afterwards in the center of the basal portion.

5. With the flattening of the culminating pseudoplasmodia, the stalk becomes continuous from apex to base.

6. The pre-spore cells are transformed very suddenly into spore cells during the early stages of culmination.

8. The fate of the spore, stalk, and basal disc cells is determined (but not irrevocably) at the onset of aggregation. Their fate is dependent on their distance from the aggregation center.

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EVIDENCE FOR THE FORMATION OF CELL  
AGGREGATES BY CHEMOTAXIS IN THE  
DEVELOPMENT OF THE SLIME MOLD  
*Dictyostelium discoideum*<sup>1</sup>

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FOUR FIGURES

INTRODUCTION

*Dictyostelium discoideum* is a member of that curious group of amoeboid slime molds, the Acrasiales, which forms one of numerous bridges between unicellular organisms and multicellular organisms. In its life cycle (see Raper, '35, '40a, '40b, '41, and Bonner, '44 for descriptive details) there is both a unicellular stage which subsequently develops by the aggregation of cells to central collection points into a differentiated multicellular organism.

So far as is known the life cycle is completely asexual. Individual capsule-shaped spore cells germinate by splitting down the side and liberating a single, uninucleate myxamoeba. This myxamoeba feeds on bacteria by phagocytosis and divides by binary fission to form many of its own kind, but each daughter myxamoeba remains a separate, independent individual. At the end of this so-called *vegetative stage*, the myxamoebae cease to feed or multiply, thus having a natural separation in their own life histories between growth processes, and purely formative, morphogenetic processes.

<sup>1</sup> This work was carried out at Harvard University, under the auspices of the Society of Fellows, to which the author wishes to express his sincere gratitude.

The myxamoebae subsequently enter the *aggregation stage* and stream in together to form a mass of cells known as a *pseudoplasmodium* (see fig. 1, 2). The pseudoplasmodium then crawls as a body for variable distances during the *migration stage*. Finally the pseudoplasmodium rights itself and rises up into the air, forming a delicate tapering stalk set at its large basal end in a small *basal disk*, and holding at its apex the *sorus* which is a spherical mass of encapsulated spores. This rise in height and differentiation of the mature fruiting body or sorocarp comprises the *culmination stage*.

*The problem that concerns us at the moment is the mechanism by which aggregation occurs; how can great numbers of independent myxamoebae be drawn together to form one unified organism.*

In a number of papers Raper ('40a, '40b, '41) reviews the past work done on aggregation and his discussions of the subject will be briefly summarized. There are 2 aspects that he has considered: the external factors affecting aggregation, and the cause of aggregation.

Many authors believe that the primary external factor involved in the stimulation of the initiation of aggregation is food shortage (Potts, '02; Oehler, '22; von Schuckmann, '24; Arndt, '37; Raper, '40b). Raper ('40b) found that the time of initiation of aggregation was shortened and the resulting patterns were made smaller by the following agents: decreased humidity, increased temperature, and light. Potts ('02) and Harper ('32) also noticed that smaller fruiting bodies were obtained in light.

Concerning the cause of aggregation, Olive ('02) and Potts ('02) independently suggested that there were chemotactic stimuli arising from the central mass of aggregating myxamoebae. Neither investigator offered any evidence for this hypothesis but believed that the general appearance indicated such a mechanism. Olive actually tried to influence aggregating myxamoebae with malic acid and sugar solutions, placed in a sealed-off capillary tube, following the work of Pfeffer on the chemotaxis of spermatozooids of ferns, but with



no encouraging results. The only other suggestion was that of von Schuckmann ('25) and Harper ('26) that aggregation is caused by a negative hygrotropic response, but Raper ('41) effectively proved that such a mechanism cannot be seriously considered.

There has been one more recent important contribution: that of Runyon ('42). He showed that, if a semi-permeable membrane of cellophane (regenerated cellulose) was placed over an aggregating pattern, and additional myxamoebae were placed on the upper side of the membrane, the upper myxamoebae would follow the myxamoebae below. The streams of incoming myxamoebae and the central collecting points would coincide above and below the cellophane sheet. Thus Runyon showed that the aggregation stimulus could pass through a semi-permeable membrane. From this he concluded that aggregation is caused by the chemotactic response of myxamoebae to a dialyzable substance.

It was thought, in the beginning of this work, that there was no real evidence that the theory of Olive, Potts and Runyon was correct for they gave no supporting evidence at all, and Runyon's ingenious experiment is hardly conclusive. A variety of physical agents besides a diffusing substance could conceivably be responsible for the orientation of the myxamoebae and could also pass through a semi-permeable membrane. But it is clear from the experimental evidence that will be presented in the following pages that the only mechanism that was supported was that of diffusion of a substance to which the myxamoebae respond chemotactically.

#### METHODS

A large part of the experimental work described here was made possible by the development of new techniques for the study of Dictyostelium. The principal of such innovations is the discovery that, contrary to Runyon's ('42) statement, aggregation will occur under water. This can be achieved by *D. discoideum*, *D. giganteum*, *D. mucoroides*, *D. purpureum*, *Polysphondylium violaceum* or *Polysphondylium pallidum* on

a water-glass interface in depths of water up to 10 cm. Depths greater than this have not been tested because there seem to be no practical or theoretical reasons for so doing. Figures 1 and 2 illustrate the appearance of this under-water aggregation. Development, however, does not proceed any further,

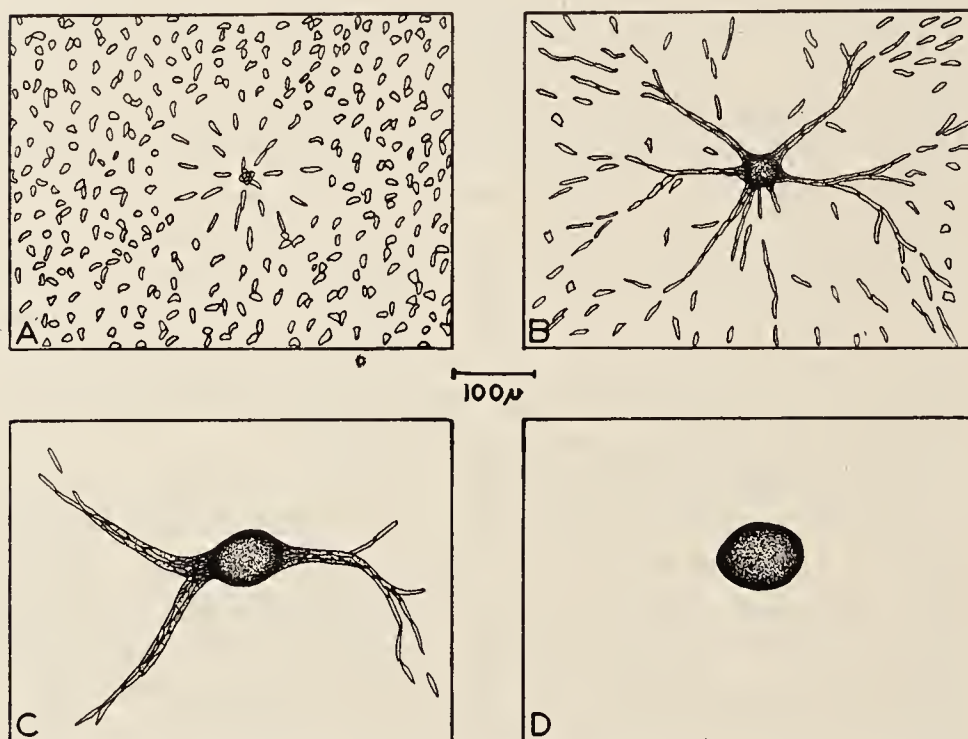


Fig. 1 A semi-diagrammatic representation of 4 stages of the aggregation of *Dictyostelium* taking place under water on the bottom of a glass dish. A, the beginning of aggregation showing the formation of a small center; B, C, successive stages of aggregation showing the thickening of the streams and the enlargement of the center; D, the final pseudoplasmodium.

leaving a rounded or irregular shaped mass of cells (see fig. 1, D). Further development (migration and culmination) can only be attained by bringing the mass into contact with an air-water or mineral oil-water interface.

Before discussing the details of this under-water technique, the standard culture technique will be described, followed by a description of the method of preparing the myxamoebae for under-water aggregation.

*Culture technique.* A large supply of myxamoebae are required for experiments on aggregation. As Dictyostelium feeds on bacteria, a large supply must be obtained and this is done by using a rich medium such as Raper ('40b) describes: (Raper's medium has been slightly modified by adding a buffer to insure a pH of about 6.0.) Peptone, 10 gm; dextrose, 10 gm;  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ , 0.96 gm;  $\text{K}_2\text{HPO}_4$ , 1.45 gm; agar, 20 gm; distilled  $\text{H}_2\text{O}$ , 1000 ml.

The inoculum of Dictyostelium spores and *Escherichia coli* (which is used as a source of food for the myxamoebae) is placed on the nutrient agar in a petri dish (90 mm diameter). The inoculum is spread over the entire surface of the agar by adding a few drops of sterile water and smearing with a sterile glass rod. The culture is incubated at room temperature for 2 days, by which time there is a thick growth of vegetative myxamoebae.

*Centrifuge technique.* In a culture which has been incubated for 2 days the myxamoebae are found spread out over the whole surface of the agar and surrounded by large numbers of bacteria. For the under-water technique the myxamoebae must be concentrated and freed from bacteria. A simple method is to wash them by centrifugation as was done by Runyon ('42) but since the details of his method have not to my knowledge, been published, the exact procedure used in this study will be described.

No sterile precautions are necessary in the centrifuge method since all nutrients are largely eliminated and there never has been any evidence of deleterious effects of contamination. The 2-day-old petri dish culture is now flooded with distilled water and thoroughly mixed with a glass rod. The suspension of myxamoebae and bacteria is placed in a centrifuge tube and centrifuged gently for 3 minutes. The force of centrifugation is regulated by a few trial experiments so that the myxamoebae will be separated from the bacteria, and thrown down to the bottom of the tube. The liquid containing bacteria is poured off, distilled water added, and the process repeated. A concentrated mass of myxamoebae rela-



tively free of bacteria is finally left in the tube to which a small amount of distilled water is again added.

*Under-water technique.* The suspension of myxamoebae is placed with a pipette directly into a syracuse or other suitable flat dish containing water. The myxamoebae soon settle to the bottom of the vessel where they subsequently aggregate. At room temperature, with optimum myxamoeba concentrations, aggregation will start in 6 to 8 hours but may be delayed by placing the dish in a cooler environment. In distilled water the myxamoebae lack adhesiveness and the slightest agitation causes them to become detached from the bottom of the dish, but if suitable electrolytes are added the myxamoebae adhere firmly to the glass substratum. This method is based on that of Mast ('29) who showed a similar effect of electrolytes on the adhesiveness of *Amoeba proteus*, and from his data the following standard salt solution (henceforth referred to as "standard solution") was devised and regularly used: NaCl, 0.60 gm; KCl, 0.75 gm; CaCl<sub>2</sub>, 0.30 gm; distilled H<sub>2</sub>O, 1000 ml. It is interesting to note that Mast showed that this is not a case of salt antagonism for the effect of the various anions is slightly additive.

*Thin film of water technique.* In a few of the experiments the myxamoebae were placed on a thin film of water on the underside of a coverslip. The procedure follows: a no. 1, 22 × 22 mm coverslip which has been carefully cleaned in 95% ethyl alcohol and wiped dry, is sealed with a mixture of approximately 2 parts vaseline and 1 part beeswax onto a van Tieghem cell (10 mm deep, 20 mm in diameter). This van Tieghem cell cup with the sealed-on coverslip serving as the bottom is filled about  $\frac{1}{3}$  full with standard solution and the myxamoebae, prepared in the fashion already described, are added. A microscope slide is then sealed over the open end of

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Fig. 2 Photographs of fixed and stained preparations showing 2 stages of the aggregation of *Dictyostelium* taking place under water on the surface of a coverslip. (Fixed in Shaudin's solution and stained by Bodian's silver impregnation method.) The upper photograph shows the beginning of aggregation with the formation of a small center; the lower photograph shows a middle stage of aggregation with a large center in the lower right hand corner.



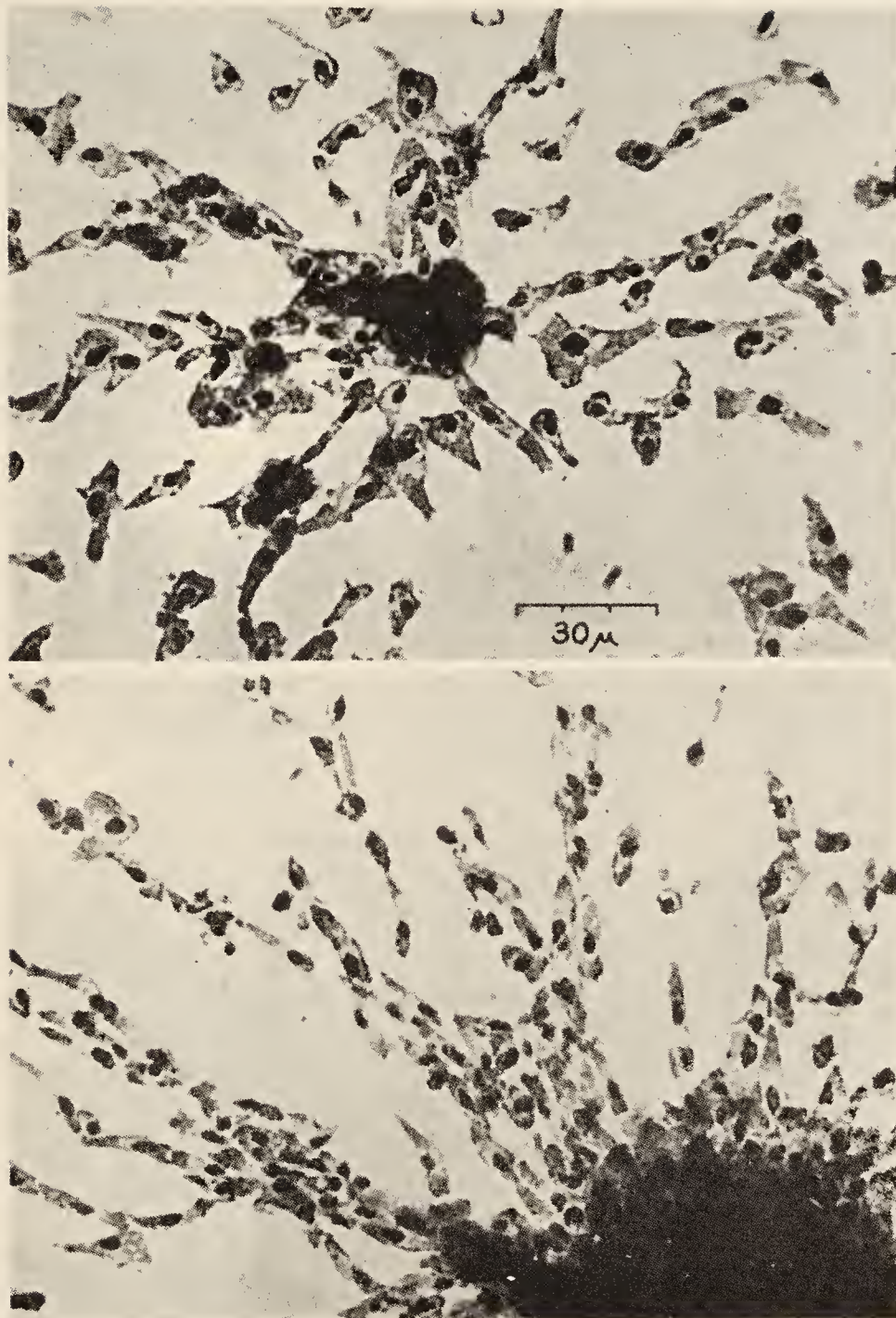


Figure 2

the van Tieghem cell. In 20 minutes time all the myxamoebae will have settled to the bottom and adhered to the coverslip. The slide is then gently turned over so that the coverslip is on the upper surface. Since the myxamoebae are in a thin film of water in contact with moist air, the organism will go through its complete life cycle producing abundant sorocarps on the underside of the coverslip.

#### RESULTS

*The attraction of myxamoebae at a distance.* That contact between myxamoebae is not a controlling factor in aggregation was first emphasized by Raper ('41). It can readily be seen in observing aggregation that isolated individual myxamoebae or groups of myxamoebae will move directly towards one of the radiating streams of incoming myxamoebae (henceforth referred to as "stream") or to the central mass of cells (henceforth referred to as "center").

With the under-water technique it was possible to get a clearer and more quantitative picture of this attraction at a distance. If one removes the center of an aggregation pattern at either of the 2 stages illustrated in figure 1, C and D, and places it beside the stream, in 3 to 5 minutes time each myxamoeba in the stream will independently turn and start to move toward the center at its new location. They will continue to move toward the center until they reach it and become incorporated into it in the normal fashion. An attempt was made to measure the maximum distance at which the center could influence the myxamoebae, care being taken to have no myxamoebae between the center and the myxamoebae under observation in order to eliminate the possibility that intermediate myxamoebae might affect the attractive power in some way. Some of the results of this type of experiment are given in table 1.

It is clear that myxamoebae up to 200  $\mu$  distant will become, in 3 to 5 minutes, oriented toward the center. If the diameter of a rounded myxamoeba is considered to be about 15  $\mu$ , then the distance between the myxamoebae and the center can be

represented as over 13 myxamoeba diameters. In a slightly modified type of experiment discussed later even larger gaps are bridged. In fact it was possible to obtain weak but definite orienting effect at a distance of  $800\mu$  or 53 myxamoeba diameters.

*The inability of an electric field to affect aggregation.* Both vegetative and aggregating myxamoebae were subjected to electrical currents of various densities. The type of chamber and the electrical circuit were essentially similar to those used by Hahnert ('32) on *Amoeba proteus*. Briefly the chamber is a small, rectangular cell ( $30 \times 10$  mm), in which the small

TABLE 1

*Table showing the ability of the centers to attract myxamoebae across various distances.*

DISTANCE BETWEEN CENTER AND MYXAMOEBAE	ATTRACTION
770 $\mu$	none
423 $\mu$	none
358 $\mu$	weak
214 $\mu$	strong
180 $\mu$	strong
128 $\mu$	strong
98 $\mu$	strong

ends are completely walled off with 2 platinum ribbon electrodes. To guard the center of the cell from harmful electrolytic products, a piece of porous material (porcelain or cellulose sponge) is placed directly in front of and parallel to each electrode. The experiments on *Dictyostelium* were done in tap water and distilled water with the same result in both cases. Parallel observations were made on *Amoeba proteus* (in tap water). The results are given in table 2. As can be seen from the table *A. proteus* shows the characteristic migration toward the cathode, while the myxamoebae of *Dictyostelium* showed no response whatever to the electrical current.

*The inability of a magnetic field to affect aggregation.* Some very cursory experiments, which are reported here for the



sake of record, were done with an Alnico magnet (1 cm<sup>2</sup> pole face) at various angles to aggregation patterns of Dictyostelium. No effect was observed.

*The inability of a conducting metal (tantalum) to affect aggregation.* This experiment is basically similar to the previously described experiment of Runyon ('42), but instead of separating the center from the myxamoebae by a semi-permeable membrane, they were separated by a thin sheet of tantalum (about 12  $\mu$  thick). A 1 cm<sup>2</sup> sheet of the tantalum was placed in a syracuse dish containing standard solution. Myxamoebae free from bacteria were allowed to settle on one side of the tantalum, using the standard technique previously described. When the myxamoebae had just started to

TABLE 2

*Table showing the effect of 3 different current densities on Amoeba proteus and on the myxamoebae of D. discoideum.*

CURRENT DENSITY IN $\mu$ AMP./MM <sup>2</sup>	EFFECT ON A. PROTEUS	EFFECT ON MYXAMOEBAE OF D. DISCOIDEUM
5	possible slight orientation	no effect
20	streams towards cathode	no effect
70	immediate death	death

aggregate, which they did normally, the sheet was turned upside down and set on a small stand (a van Tieghem cell 5 mm deep, 20 mm in diameter) so that it was not touching the bottom of the dish and yet was completely submerged in the standard solution. A large active center from another dish was taken in a micro-pipette and placed on the upper surface of the tantalum.

In no case was the effect of the upper center transmitted through the sheet, and the myxamoebae below, while they aggregated normally, bore no relation in their pattern to the strong center above.

*The impermeability of glass, mica, and quartz to the aggregation stimulus.* Using the same technique as described immediately above, coverslip glass (120  $\mu$  thick), mica (100–

150  $\mu$  thick), and quartz glass (50–100  $\mu$  thick) were tested. Again, in no case was there any visible orienting effect transmitted through these materials although aggregation appeared normal in each instance.

*The attraction of myxamoebae around corners.* This experiment was designed to see if a center could orient myxamoebae that were not in a direct line with the center, but were separated by an impermeable substance that could be circumvented. Some myxamoebae free of bacteria were spread on a no. 1 coverslip (22  $\times$  22 mm, approximately 160  $\mu$  thick) which had been placed in the bottom of a syracuse dish full of standard solution. When the myxamoebae had just begun to aggregate the coverslip was turned upside down and held in such a position so that it formed a shelf, completely surrounded by standard solution. This is represented diagrammatically in figure 3, A. An active, strongly attractive center was placed approximately 60  $\mu$  from the edge of the coverslip, on the upper surface. Very shortly afterwards, the separate myxamoebae on the underneath surface became oriented towards the point on the edge nearest the center above and moved up around the edge to join the center (see fig. 3, A). In other words the center exerted its influence around the corner, from the upper surface to the lower surface.

*The inability of the orientation of the substratum to affect aggregation.* The following experiments involve using the techniques of Weiss ('45) who obtained oriented growth of fibroblasts of chick embryos by placing them on specific types of substrata. Myxamoebae were placed on a sheet of mica in which shallow grooves had been scratched with a fine steel needle (see Weiss, '45 for the details of the preparation of the mica). Neither the aggregating myxamoebae, nor the wandering vegetative myxamoebae showed any preference for the grooves, but would pass across them as though completely unaffected by their existence. Glass fibers (from glass wool) lying in a heap under water in a syracuse dish were also covered with myxamoebae and again the myxamoebae showed



no more tendency to adhere to the fibers than to the glass bottom of the dish.

*Attempts to observe structural connections between aggregating myxamoebae.* It is not possible in a living preparation, even using an oil immersion (1.8 mm) objective and preparing the material with the thin film of water technique, to see any

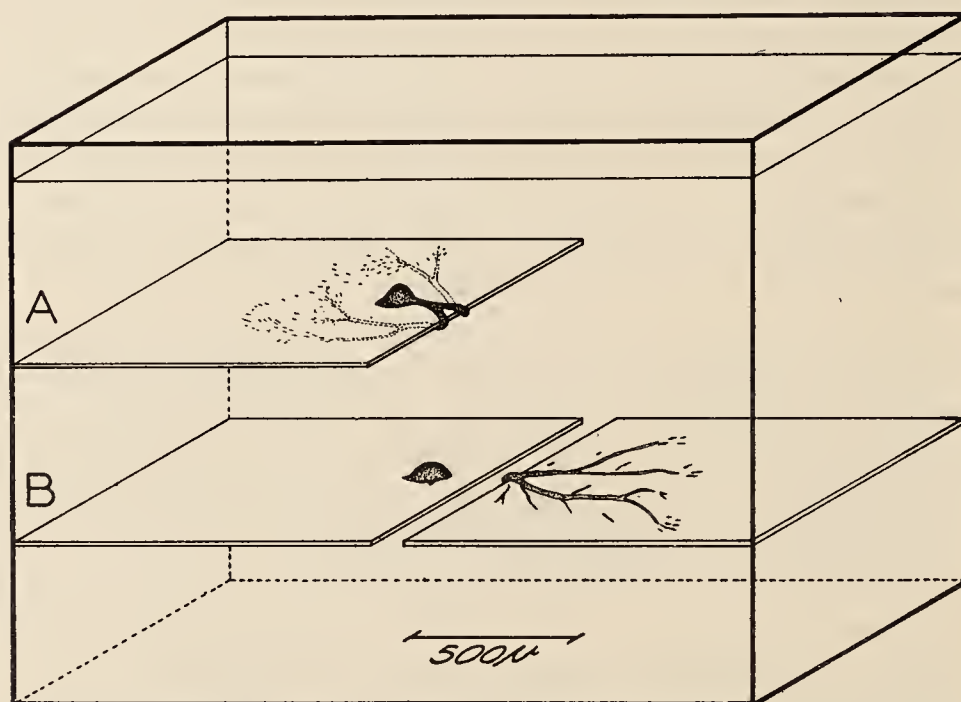


Fig. 3 A semi-diagrammatic representation of 2 experiments done on aggregation in *Dictyostelium* using coverslip shelves held under water. A, the myxamoebae previously at random under the coverslip are attracted around the edge to the center on the upper surface; B, the myxamoebae previously at random on the right hand coverslip are attracted to the center on the left hand coverslip, across the substratum gap.

connections between the aggregating myxamoebae except when the cells are half a cell diameter or less from one another. Then they often are attached by definite filopodia. To examine this point further, aggregation stages were stained with silver using the technique of Bodian. Aggregation was allowed to occur on coverslips under water and at the desired stages they were removed, fixed in Shaudin's solu-

tion, and stained. The gold toning was not used. Photographs of such a preparation are shown in figure 2. As can be seen from the photographs, and also from careful oil immersion examination of the slides, there is no evidence of any filopodia extending any great distances. Nor is there any evidence of any material, exudate, or ground mat such as Weiss ('45) describes for oriented chick fibroblasts, which he stained in the same fashion.

*An attempt to reveal a deposit made by aggregating myxamoebae that might orient other myxamoebae.* It was possible to show by experiment that an aggregation pattern leaves behind no structure on the substratum that can orient myxamoebae. The standard solution was poured off a syracuse dish in which the aggregation was complete or nearly so for all the pseudoplasmodia. The dish was then placed in the ice compartment of an electric refrigerator and allowed to freeze. After removal from the refrigerator more standard solution and fresh myxamoebae were added. The old centers that had been killed by freezing could still be seen. Care was taken to observe if the live myxamoebae were affected in any way by any type of structure that the previous aggregation patterns might have created. No such effect was demonstrated; the new patterns bore no relation to the previous ones.

*The importance of an interface connecting the center and the myxamoebae.* It was found that a center could attract myxamoebae to which it was not directly connected by an interface. This fact was first realized in an accidental observation. A hanging drop preparation had been made with myxamoebae free from bacteria in standard solution and a clear aggregation pattern formed at the base of the drop, at the air-water interface. I noticed that above this pattern, on the glass-water interface, a few myxamoebae were aggregating to a center directly above the center of the aggregation pattern below. Since the upper center formed at the nearest point to the lower center, one can reason that this was caused either by the fact that the positions of both the upper and

lower centers were determined by similar tensions in the drop, or that the lower center, which was the farthest advanced of the two, directly influenced the upper myxamoebae without being directly connected to them by an interface.

In an effort to rule out the possible effect of the tensions in a hanging drop, an experiment was designed in which 2 no. 1 coverslips ( $22 \times 22$  mm) were prepared in standard solution, one with many myxamoebae just starting to aggregate, and the other with one large center. One was then turned over and placed on top of the other, taking care to prevent their surfaces from touching by placing 2 wedges between them, one on each side. The myxamoebae were then facing the center, separated by a thin layer of standard solution. Again an immediate response was obtained and the myxamoebae formed a pattern so that their center was directly opposite, that is the shortest distance from, the strong center on the opposite coverslip. Numerous attempts were made to determine how far apart the plates could be and still obtain an orienting influence of the center on the myxamoebae. If the plates were  $500 \mu$  apart the effect was obvious and strong. At  $800 \mu$  there was a weak and diffuse, but still discernible orienting effect. Thus without the disturbing surface tension effects of a hanging drop, a center again affected distant myxamoebae to which it was not directly connected by an interface.

This was done in another striking way illustrated in figure 3, B. Two coverslips were prepared as above and placed side by side in a dish to form 2 shelves, surrounded by standard solution, and separated by a small gap. The center was placed fairly near the edge, and it immediately affected the myxamoebae on the opposite shelf so they all streamed to the nearest point on the edge (see fig. 3, B). Again the effect of a center was transmitted across a gap which possessed no interface, but merely a layer of water.

A few observations on this experiment should be mentioned. The first myxamoebae that got to the edge appeared to be reaching out into the gap between the coverslips with a sort of hopeless pseudopodial waving. Later when they became



more numerous, they formed their own center directly opposite the original center on the other coverslip. However, if the coverslips were closer together so that the gap was very small (about 20–30  $\mu$ ) then the myxamoebae formed a stream, a bridge right across the gap and joined the center on the opposite side.

*The effect of water flowing over aggregation patterns.* A flow of water was created over myxamoebae that were about to aggregate or that were in the process of aggregating and a distinct modification of the aggregation pattern was obtained. The flow of water was achieved 2 different ways. In one a glass rod (4 mm in diameter) bent into an "L" shape was attached to a shaft of a 6 RPM electric motor, and held over the center of a syracuse dish (containing bacteria-free myxamoebae) so that the lower bar of the "L" was just submerged in the water, forming a radius to the circular dish. In this way the water was slowly swirled in a circular fashion, creating a fairly linear flow at any spot in the dish other than near the center. The other method of creating a flow involved drawing water through a channel between 2 coverslips (approximately 100  $\mu$  apart). A controlled rate of flow was obtained by leading the water from a reservoir to the coverslips through a fine glass capillary tube.

If the myxamoebae were about to aggregate when placed in this current, they continued to do so, but the aggregation patterns, as shown in figure 4, A, were atypical. Each possessed only one unusually long stream which always approached the center from the downstream side. If the myxamoebae had already started normal aggregation before being subjected to the flow, they would quickly assume a similar form as shown in figure 4, B. The streams that had existed on the upstream side would break up rapidly, and although the downstream and lateral streams remained, they would continue to form only in line with the direction of the flow of water. In both cases the most striking fact was that the myxamoebae, upstream of the center, even those almost touching it, showed no effect of any stimulus from the center

and moved in a random fashion. Yet judging from the length of the streams, the stimulus from the center apparently had extended an abnormally long distance downstream. If vegetative myxamoebae were placed under such a current no effect was observed whatsoever, but they continued their random locomotion.

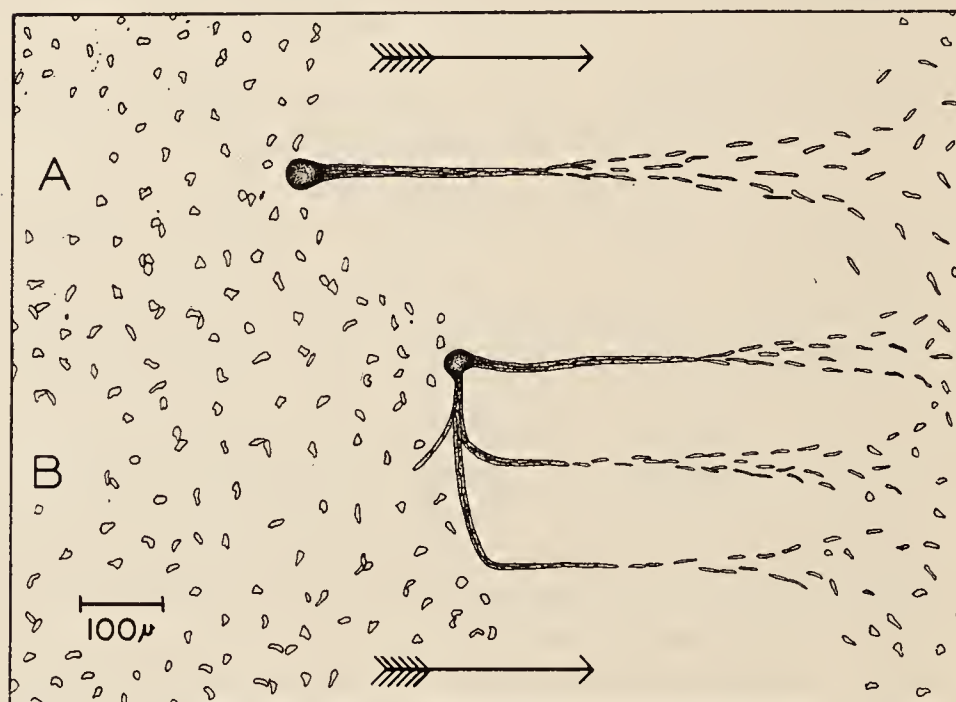


Fig. 4 A semi-diagrammatic drawing showing the effect of a moving stream of water (the arrows indicate the direction of movement) over an aggregation pattern in *Dictyostelium*. A, an aggregation pattern that formed while the water was in motion; B, an aggregation pattern that formed in still water and was subsequently subjected to a stream of moving water.

#### DISCUSSION

If the facts that have been obtained from the experiments described above, and those from the literature are summarized, the following statements can be made of the aggregation process in *Dictyostelium*: (1) the attraction can operate across a semi-permeable membrane (Runyon, '42); (2) the center will attract myxamoebae at considerable dis-

tances; (3) it has been impossible to date to show any effect of an electric field or (4) of a magnetic field on the aggregation pattern; (5) aggregation occurs on the surface of a conducting metal (tantalum), but (6) a center will not attract myxamoebae when separated from the myxamoebae by a sheet of tantalum; (7) nor can a center attract myxamoebae through glass, (8) mica, or (9) quartz; (10) a center can attract myxamoebae around a corner of some impermeable substance; (11) the structure of the substratum does not appear to affect the aggregation pattern; (12) it has been impossible to demonstrate any sort of bridge or connection between a center and distant myxamoebae; (13) there is no evidence of any myxamoeba-orienting substance deposited on the substratum; (14) a center does not have to be directly connected to myxamoebae by an interface in order to attract them; (15) aggregation patterns forming under flowing water are deformed so that only the myxamoebae directly downstream of the center will show any orientation.

In our search for the immediate cause of aggregation in *Dictyostelium*, the first fundamental question that arises is, does the center pull in the myxamoebae by force or does it merely orient the myxamoebae by stimulation? There is every indication that the energy of locomotion is contributed by each individual myxamoeba, and that the center orients them by a differential stimulation.

The problem might be approached by examining various reasonable possible mechanisms suggested by investigations on other forms in the light of the facts known about aggregation in *Dictyostelium*. For instance one might consider the likelihood of aggregation to be: (1) an agglutination process; (2) in some way controlled by an electric or (3) magnetic field; (4) or by some form of radiation; (5) controlled by some type of orienting structure deposited on the substratum; (6) or by the oriented molecules in a molecular surface film; (7) or, finally, controlled by the gradient of a substance to which the myxamoebae respond chemotactically.



*Agglutination hypothesis.* Some actual immunological work has been done on *D. mucoroides* by von Schuckmann ('25) who showed that when rabbit antiserum to vegetative myxamoebae is added to a culture of similar myxamoebae, they will agglutinate into clumps in an irreversible fashion. Unfortunately von Schuckmann does not describe the details either of the process or of further development, if any, of the clumps; but it is notable that he does not attempt to interpret normal aggregation in terms of agglutination. In some work that will be reported in detail at a future date, I have been able to obtain pseudoplasmodium formation by what is apparently agglutination, and these pseudoplasmodia develop into complete sorocarps. But in such an instance the normal aggregation process has been completely circumvented and the pseudoplasmodium has been achieved by an unnatural means.

There are a number of strong evidences against the "clumping" of myxamoebae found in normal aggregation being an agglutination process. In the first place the fact that a center can attract myxamoebae across great distances is inconsistent with agglutination. A basic property of all agglutination processes is that the cells come together by chance collision; for example as a result of active random motion or brownian motion, followed by a firm adhesion of the collided cells.

Another evidence comes from the fact that the myxamoebae which are in contact can and do normally separate readily from one another. A striking example of this is the case mentioned where a center is removed and placed laterally to its stream. The compacted mass of myxamoebae forming the stream will break up, each myxamoeba becoming unattached and going independently to the new location of the center. Such a phenomenon is never seen in agglutination processes. The nearest they approach it is in the case of reverse agglutination, but then up to the point of reversal the cells are solidly stuck to one another. In *Dictyostelium* the myxamoebae can be separated at all times.

A final evidence that aggregation is not achieved by agglutination comes from the fact that attraction between the center and the myxamoebae is obtained through a semi-permeable membrane as previously described in the experiment of Runyon ('42). Proteins or complex polysaccharides are the important molecules in immunological reactions, and even the smallest proteins will not (except possibly at an extremely slow rate) pass through the regenerated cellulose dialyzing membranes used. I have repeated Runyon's experiments many times with different types of membranes all presumably impermeable to proteins, and always obtained the same sort of rapid (3 to 5 minute) response of the myxamoebae to the center on the opposite side of the membrane. From these arguments it may be concluded that aggregation in Dictyostelium is not an agglutination process. This does not exclude the possibility that antigen-antibody reactions are factors in development at a later stage. But some other factor must be responsible for initiating the normal "clumping" of the Dictyostelium myxamoebae.

*Electrical hypothesis.* The evidence weighs against the possibility that aggregation can be explained in terms of an electric field. Consider first the fact that an electric field surrounding aggregation patterns will not affect them. While it is true that such negative evidence is in no way conclusive, it is nevertheless indicative.

Perhaps better evidence comes from the tantalum experiment, where normal aggregation was found to occur on the surface of a conducting metal, but the attraction could not take place through a thin sheet of the metal. If the aggregation mechanism were electrical then the metal would most likely have created a short circuit preventing aggregation, which is not the case. Even granting that aggregation could occur on the surface of the metal, one would further expect the attraction to be conducted through the tantalum sheet.

*Magnetic hypothesis.* Magnetism is also an unlikely possibility for reasons very similar to those mentioned for electricity. Not only was the aggregation pattern not affected by

a magnetic field (which again is only indicative evidence) but also the attraction could not pass through a tantalum sheet, which could be readily achieved by a magnetic force.

*Radiation hypothesis.* Rays emitted from the center of Dictyostelium could conceivably guide the myxamoebae, but the evidence does not support this view. First, if such a ray exists, it can penetrate water, and cellophane, but is stopped by glass, quartz and mica. This certainly does not eliminate the hypothesis but it narrows the possibilities.

Stronger evidence against a radiation phenomenon comes from the experiment in which a center on the upper surface of a coverslip attracted myxamoebae on the lower surface, around the edge (fig. 3, A). It is difficult for me to see how a ray mechanism could operate here for if the coverslip were permeable to the ray one would expect the underneath myxamoebae to aggregate to the point nearest the above center, which would be directly below the center on the underside of the glass. If the coverslip were impermeable to the hypothetical ray, then one would expect no effect whatsoever of the upper center on the myxamoebae underneath. But this is not the case; the myxamoebae go around the edge. Rays travel in straight lines and can hardly be expected to pass from one side of an impermeable barrier to the other.

*Contact guidance hypothesis.* "Contact guidance" designates the idea that the amoeboid processes of cells are oriented by being mechanically guided by either the ultra-structure of the substratum or the direction of flow of an exudate given off by the cell. The concept of contact guidance is that of Weiss ('29, '34, '45) who studied the causes of orientation in cell growth and movement of cells of higher animals in tissue culture.

There is no evidence to indicate that contact guidance plays a part in aggregation for it has been impossible to demonstrate any guiding structure deposited on the substratum, or even that the orientation of the substratum itself has any orienting effect.



*Molecular surface film hypothesis.* It was thought, since aggregation only occurs at an interface, that a molecular surface film might be involved in orienting the aggregating myxamoebae. But this hypothesis was invalidated by showing that it is quite possible for a center to attract myxamoebae when the center is not connected directly to the myxamoebae by an interface.

#### CONCLUSION

*Diffusion hypothesis.* Good evidence that aggregation is achieved by the center producing a substance to which the myxamoebae respond chemotactically<sup>2</sup> comes from the experiment in which the aggregation patterns are deformed by flowing water. In fact the following deductions can be made from this experiment: (1) No mechanical explanation, such as the direct effect of the current on the myxamoebae, could explain the patterns obtained under flowing water because vegetative cells in the current continued normal random movement. That is, the pattern was not imposed on the myxamoebae by the external moving water, but it arose in the normal fashion as a result of the activities of the myxamoebae themselves. (2) Under the flow of water the center remained the source of production of the stimulating agent for the myxamoebae were attracted to it. (3) The agent was washed downstream — the myxamoebae upstream were not attracted in any way by the center, whereas the myxamoebae downstream were attracted to it from great distances. (4) The only reasonable type of agent that could be carried along in such a fashion by a slow current is a free-diffusing chemical substance. (5) The fact mentioned previously that a substance can only be effective in orienting the myxamoebae when it is

<sup>2</sup> A note should be made here concerning the use of the word chemotaxis in this paper. As Blum ('35) points out there are 2 separate factors to consider in oriented movements; (1) the orientation of the organism, in this case to the diffusion field of a chemical substance and (2) the movement of the organism which may quite possibly be in no way affected by the chemical substance (since there is movement in the absence of the substance).

in a gradient helps interpret a number of phenomena. For instance: the myxamoebae upstream must have been surrounded by the substance which came from the other centers (and let us assume it is in a high enough concentration to be able to obtain a response) but they showed no orientation because the substance in that region became, by diffusion, more evenly distributed and not in a sufficient concentration gradient to cause orientation. (6) Also the only method in which a gradient could be maintained during aggregation would be by a constant production of the substance of the center. Since diffusion would tend always to obliterate the gradient, and the maintenance of a steady state is necessary, it must be assumed that the substance is produced either continuously or at frequent intervals by the center.

Therefore in summarizing we have deduced from this flowing water experiment that during the aggregation of *Dictyostelium* there is some type of chemical substance (which is not necessarily homogeneous but might consist of a group of compounds) produced continuously or at frequent intervals by the center, which freely diffuses, and the myxamoebae move in the resulting gradient of this substance towards the point of its highest concentration. The final proof of the existence of the substance (and an important problem for future research) must be its isolation *in vitro*. But considering the present weight of evidence, it seems fitting to propose tentatively a name for the substance. The term *acrasin* is suggested, and it can be defined for the moment as a type of substance consisting either of one or numerous compounds which is responsible for stimulating and directing aggregation in certain members of the Acrasiales. It also may perform other duties in the development of these organisms but such considerations are not within the scope of this paper. Also at a later date I plan to present an examination of the formation of streams during aggregation, a process which may appear puzzling in the light of the present discussion.

One of the most difficult factors to understand in any chemotaxis hypothesis is how it is possible for a single small

amorphous amoeboid cell to be sensitive to gradients of diffusing substances. The concentrations of these substances in a great many of these cases must be small, and of course, the molecules in diffusion move at random in all directions. Thus the cell must detect very small concentration differences between one end of the cell and the other.

In an attempt to calculate just what this concentration difference would be between the ends of a myxamoeba in the gradient of a hypothetical substance, Dr. L. J. Savage has been kind enough to derive and evaluate the following approximation (see appendix):

$$-\frac{\Delta c}{c} \approx \frac{\Delta r}{r}$$

where  $c$  is the concentration at any part of the myxamoeba,  $r$  is the distance from any part of the myxamoeba to the mid-point of the bottom of the center,  $\Delta c$  is the difference in concentration between the far and near ends of the myxamoeba, and  $\Delta r$  is the length of the myxamoeba. If we choose some reasonable values, letting  $\Delta r = 25 \mu$ ,  $r = 500 \mu$  they can be substituted in the above expression:

$$\begin{aligned} -\frac{\Delta c}{c} &\approx \frac{25}{100} \\ -\Delta c &\approx .05c \end{aligned}$$

Thus the concentration difference between the 2 ends of a myxamoeba at  $500 \mu$  distance from the center would be 5% of the total concentration. Remember that a gap much over  $800 \mu$  can no longer be bridged, which would mean that if the concentration difference of the hypothetical substance across a myxamoeba is less than 3%, it no longer would be effective in orienting the myxamoeba. These values appear sufficiently high so that from this point of view the chemotaxis hypothesis is not unreasonable.

#### SUMMARY

*Dictyostelium discoideum* is a member of that group of amoeboid slime molds (Acrasiales) characterized by forming



the fruiting structure from a compacted mass of uninucleate myxamoebae known as a pseudoplasmodium. The pseudoplasmodium arises by the aggregation of many myxamoebae which were previously completely independent and separate from one another. In this so-called aggregation stage radial streams of elongate myxamoebae move towards a central point by means of pseudopodial locomotion. Attempts to discover the immediate cause of this centripetal streaming of myxamoebae revealed that it is not an agglutination process; that a spreading molecular surface film phenomenon is not responsible; that an electrical or magnetic force is improbable; that any form of directing ray is not involved; and that no type of predetermined structural matrix exists that could guide the myxamoebae to the center. However, evidence was obtained for a substance diffusing from a central mass of myxamoebae through the liquid medium and the incoming myxamoebae orienting themselves in the diffusion field, moving towards the point of highest concentration. This was shown by inducing a gentle stream of water to flow over an aggregation pattern, causing in the usually radial pattern of incoming myxamoebae an asymmetry that can only be interpreted as the warping of the diffusion field of a substance to which the myxamoebae are sensitive. This type of substance has been tentatively called *acrasin*.

#### APPENDIX

*The derivation and evaluation of the approximation used to calculate the difference in concentration of acrasin between the 2 ends of an aggregating myxamoeba.* By L. J. SAVAGE.

If a hemispherical source resting on the bottom of a large tank produces  $Q$  gm/day of a stable compound with diffusion coefficient  $D$  cm<sup>2</sup>/day, then (after the source has been in position for some time) the concentration  $c$  of the compound at the distance  $r$  from the center of the base of the hemisphere is given by

$$c = \frac{Q}{2\pi D} \frac{1}{r} \quad (1)$$

Equation 1 is derived in essentially the same way as equation 20 on page 17 of *Mathematical Biophysics* by Rashevsky ('38).

Let  $r_1$  and  $r_2$ ,  $r_1 > r_2$ , be any 2 different values of  $r$ ,  $\Delta r = r_1 - r_2$ , and  $\Delta c$  be the difference between the concentrations at  $r_1$  and  $r_2$ . Then if  $r$  is between  $r_1$  and  $r_2$  and  $c$  is the concentration at some point  $r^1$  also between  $r_1$  and  $r_2$  we have from equation 1.

$$-\frac{\Delta c}{c} = r^1 \left( \frac{1}{r_2} - \frac{1}{r_1} \right) = \frac{\Delta r}{r} \frac{rr^1}{r_1 r_2} \quad (2)$$

From equation 2 we conclude that if  $\Delta r$  is small compared with  $r_2$ , then

$$-\frac{\Delta c}{c} \approx \frac{\Delta r}{r} \quad (3)$$

More precisely, we evaluate  $-\Delta c/c$  thus:

$$-\frac{\Delta c}{c} \leq \frac{\Delta r}{r} \frac{r_1^2}{r_1 r_2} = \frac{\Delta r}{r} \frac{r_1}{r_2} = \frac{\Delta r}{r} \left( 1 + \frac{\Delta r}{r_2} \right), \quad (4)$$

and

$$-\frac{\Delta c}{c} \geq \frac{\Delta r}{r} \frac{r_2^2}{r_1 r_2} = \frac{\Delta r}{r} \frac{r_2}{r_1} = \frac{\Delta r}{r} \left( 1 + \frac{\Delta r}{r_2} \right)^{-1} \quad (5)$$

Summarizing inequalities 4 and 5

$$\frac{\Delta r}{r} \left( 1 + \frac{\Delta r}{r_2} \right)^{-1} \leq -\frac{\Delta c}{c} \leq \frac{\Delta r}{r} \left( 1 + \frac{\Delta r}{r_2} \right) \quad (6)$$

by way of a numerical example, suppose  $r_2 = 585 \mu$ ,  $\Delta r = 30 \mu$ , and  $r = r^1 = 600 \mu$ , then  $\Delta r/r = .050$  and inequality 6 guarantees that  $.0476 \leq -\Delta c/c \leq .0526$ .

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## This Week's Citation Classic

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**Bonner J T.** Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*.  
*J. Exper. Zool.* 106:1-26, 1947. (With an appendix by L J Savage.)  
[Harvard University, Cambridge, MA]

The amoebae of cellular slime molds first undergo growth as separate cells and then aggregate to form cell masses that become differentiated multicellular organisms. This paper gave evidence that aggregation occurred by chemotaxis, and the chemical attractant was given the name *acrasin*. [The *SCI*<sup>®</sup> indicates that this paper has been cited over 185 times since 1961.]

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December 1, 1978

"It is difficult to believe today that it was once necessary to prove that cellular slime molds aggregated by chemotaxis. In the 1940s it was thought that most morphogenetic processes, which often had been assumed to be chemotactic, were probably not so, and could be explained by other mechanisms such as 'contact guidance.' Chemotaxis had first been postulated for cellular slime molds by G. Potts<sup>1</sup> in 1902, but it was not until the earlier work of Ernest Runyon<sup>2</sup> and this paper that the idea became accepted. My research was done at Harvard after I left the Army to finish my graduate studies; the paper was my Ph.D. thesis.

"Since 1947, there has been an enormous increase in the interest in the development of the cellular slime molds. At that time there were a mere handful of workers; today there are probably two hundred scattered over the world. It is conceivable that this paper played some part in bringing

slime molds to the attention of developmental biologists, especially those who had been trained as molecular biologists on *E. coli* and were looking for a eukaryote that had an uncomplicated development. But there are many other papers that also contributed, especially those of Kenneth Raper<sup>3</sup> in the early 1940s; his certainly were the ones that trapped me.

"If my paper has any importance, this may be the reason, but unfortunately it has nothing to do with why it is cited so often. The explanation for its popularity is that in the 'methods' section I devised a physiological salt solution (based on S.O. Mast's work<sup>4</sup> on *Amoeba proteus*) which has been used by many workers in the field. It is a simple mixture of NaCl, KCl, and CaCl<sub>2</sub> which apparently keeps the amoebae in a particularly happy and healthy state in most (but not all) circumstances. When it first was used by others, I was greatly elated by the fact that they referred to it as 'Bonner's solution.' What could be more impressive than having a 'solution' bearing one's name? Unfortunately, this dignified label has quite disappeared and now it is often called 'Bonner's salts,' which raises a different picture in my mind, something one might need a 'dose of.' To compound this unhappy trend, I occasionally find bottles in our laboratory refrigerator labeled 'BS;' it gives me a feeling that one of my graduate students is sending me a message. But, on the whole, and despite these problems, I do not mind assuming the role of the Fanny Farmer for slime molds; there are worse fates."

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A STUDY OF THE CONTROL OF DIFFERENTIATION: THE PROPORTIONS  
OF STALK AND SPORE CELLS IN THE SLIME MOLD  
*Dictyostelium discoideum*<sup>1</sup>

John Tyler Bonner and Miriam Kresses Slifkin

THE PROCESS of differentiation has two aspects; the arising of differences in protoplasmic structure, and the arrangement of these differences in space to form a pattern. In most cases this pattern is achieved with the help of growth, making it difficult to assess and study the role played by the differentiation process itself, but in the amoeboid slime mold *Dictyostelium discoideum* it is possible to observe and study the contribution which differentiation alone makes to the pattern.

In the first place growth in this organism is completely separate from the morphogenetic stages of the life cycle, for the myxamoebae divide and feed separately and independently in the vegetative stage, then cease all growth processes and aggregate to form sausage-shaped migrating pseudoplasmodia. After a period of wandering these pseudoplasmodia turn up into the air and develop into a delicate stalk made up of large vacuolated myxamoebae, and an apical spore mass, each capsule-shaped spore containing a single uninucleate myxamoeba (Raper, 1935; Bonner, 1944).

As was pointed out previously (Bonner, 1944) the pre-spore and pre-stalk cells are already recognizable in the latter part of the migration stage. The pre-stalk cells are anterior in the migrating pseudoplasmodium and are larger than the smaller dense posterior pre-spore cells. The line of demarcation between these two cell types was shown to be very sharp. The subsequent development in-

volves morphogenetic movement and the final differentiation into stalk and spore cells.

The location of the line of demarcation between the pre-spore and the pre-stalk cells determines the proportions. In other words, the relative number of stalk cells and the relative number of spore cells is established without growth and in a mass of cells all potentially similar, differing only in their relative position in the whole pseudoplasmodium.

The purpose of this paper is to obtain information that might ultimately lead to understanding the mechanism of the control of differentiation. Two approaches have been used: one is the study of environmental factors which might possibly affect the proportions, and the other is a search for possible connections between acrasin (Bonner, 1947, 1949) and the process of differentiation.

MATERIALS AND METHODS.—*Technique of preparing total mounts.*—Hitherto it has been impossible to obtain total mounts of the Acrasiales without losing the majority of the spores which are very loosely bound in the sorus. Since total mounts are essential if the sorus volume is to be measured, a technique was devised for this specific purpose.

A petri dish bottom containing a mature culture was inverted over a petri dish cover containing full strength formalin solution. The solution was heated, by means of a hot plate, to approximately 35°C. for 10 min., and then allowed to cool to room temperature. A small circle of the agar containing fruiting bodies was then cut out with a glass ring, and the agar was transferred to a 22 mm. square cover glass. The cover glass with the agar adhering

<sup>1</sup> Received for publication July 1, 1949.

This work was carried out with the help of a grant from the American Cancer Society.



to it was held, agar surface down, in Schaudinn's fixative (containing also one drop of 25 per cent aerosol solution) for 1 min. and then turned right side up and allowed to sink to the bottom of the Schaudinn's where it remained for 10 min. After the fixation the agar blocks received the following treatment: 50 per cent ethyl alcohol for 5 min.; distilled water for 5 min.; stained for 20 sec. in 1 per cent aqueous solution of Bismarck Brown; washed 5 min. in distilled water; in 50 per cent glycerine—50 per cent distilled water for 30 min. All the above steps were done in Columbia staining dishes and the solutions were carefully poured into the dishes to avoid damaging the sorocarps or freeing them from the agar. Finally, each fruiting body was mounted on a slide which had been previously ringed with gold size to serve as a washer to keep the coverslip from pressing against the sorocarps. Glycerine jelly was used as a mounting fluid and for permanence the coverslips were also ringed with gold size.

*Methods used to estimate the volume of the stalk and sorus.*—The segment method.—A camera lucida was set in such a way so that the drawing was 1000 times larger than the object. The entire sorus was drawn, but since the stalk was too long to fit in the field its width was drawn at 200  $\mu$  intervals along the entire length of the stalk. Thus the stalk was already divided up by a series of equidistant, parallel lines, and the same was done on the drawing of the sorus. Then the volume (V) for each segment of both sorus and stalk was calculated as if its ends were circular and the surface between corresponding points lay along a line (just as in a segment of an ordinary circular cone, for example). The formula used was

$$V = \frac{h\pi}{24} (D_a^2 + (D_a + D_b)^2 + D_b^2)$$

where h is the distance between the two limiting parallel planes, and  $D_a$  and  $D_b$  the diameter of these planes (which are assumed to be circular).<sup>2</sup>

The rapid method of volume measurement using the Turrell (1946) tables.—It was found necessary

to develop a more rapid method of volume estimation and the following procedure was found satisfactory. In the sorus only the lengths of the major and minor axes were needed to obtain the volume from Turrell's tables, for the sorus is a prolate spheroid. Since sori closely resemble lemons in relative proportions (and incidently Turrell's tables are designed for measuring the volume of lemons), a dozen lemons were measured for their actual volume by water displacement. Also with shadow pictures, their volumes were estimated by the segment and Turrell methods. The segment method gave an average of 5 per cent smaller volumes than shown by water displacement, and the Turrell method gave an average of 9 per cent smaller volumes, indicating that all these are reasonably close.

In this rapid method (which for convenience we will call the Turrell method) the stalk volume was estimated by the following simple method.

$$V = \frac{\pi h}{4} D_o^2$$

where  $D_o$  is the diameter of the stalk at its middle. In order to equate the Turrell method with the segment method eighty-one sorocarps were measured by both methods, and each was expressed in terms of the ratio of sorus to stalk volume. A curve was drawn through the points and from this one could readily convert the Turrell data into the terms of the more precise segment method.

*RESULTS.—The proportions of individuals fruiting at 17°C. in the dark.*—The first step in this study of proportions was to examine a large series of organisms in one environmental condition in order to obtain an estimate on the variability and to find whether or not the proportion varied with the size of the pseudoplasmodium.

After inoculation the amoebae were grown for 2 days in diffuse light at room temperature (21–

<sup>2</sup>The authors are extremely grateful to Donald T. Chalkley of this laboratory for his helpful advice on this method, and for letting us use his tables which were a tremendous time saver since one could obtain the volumes directly, knowing the values for h,  $D_a$  and  $D_b$ .



24°C.) and then just prior to aggregation they were placed in the dark at 17°C. Stained total mounts were made of fifty-six individual slime molds and they were carefully measured using the segment method of approximating volumes. The sorus volume is plotted against the stalk volume in fig. 1<sup>3</sup>.

Since in fig. 1 the relation between sorus and stalk volume is a linear one, and since the total number of cells varies greatly, the proportions are independent of the total size of the pseudoplasmodium.

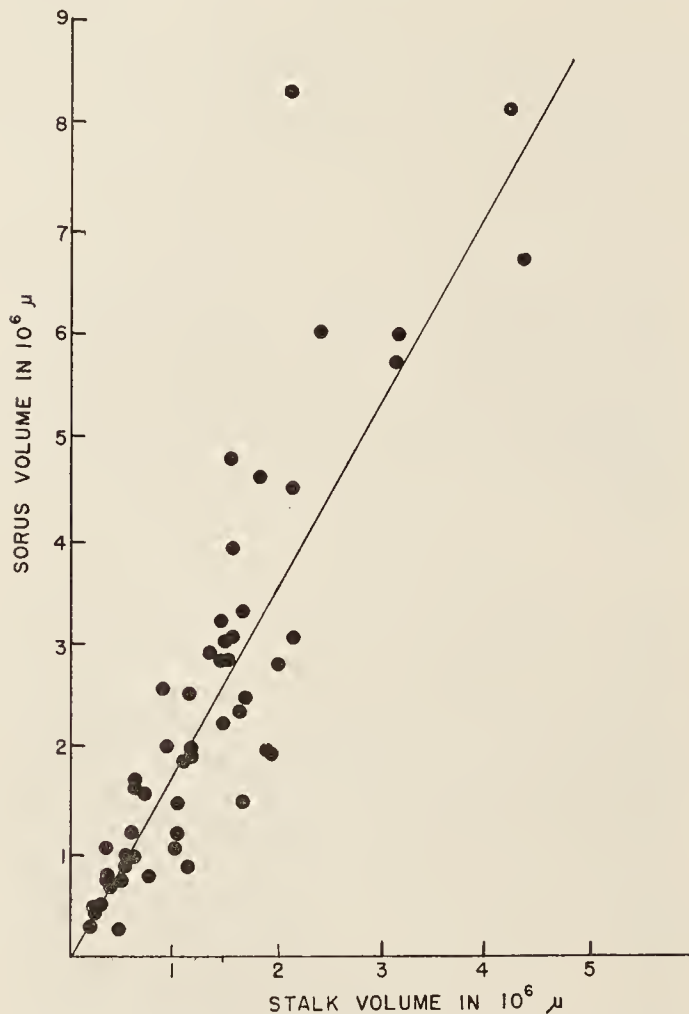


Fig. 1. Graph showing the relation between the volume of the sorus and the volume of the stalk for sorocarps fruiting at 17°C. in the dark.

If the volumes are converted to cell numbers using the standard conversion figures,<sup>4</sup> a mean of 14.1 per cent of the cells are stalk cells and the remaining 85.9 per cent spore cells. The standard deviation for the 14.1 per cent stalk cells is  $\pm 6.26$

<sup>3</sup> No attempt has been made in this study to measure the volume of the basal disc. The number of cells involved in the basal disc is relatively small and for technical reasons it was found too difficult to measure them with any accuracy.

Unless otherwise specified the organisms used had fruited for a number of generations at 17°C. The importance of this will be seen shortly.

<sup>4</sup> It was determined that the number of stalk cells per  $(10\mu)^3$  was approximately 4.3, and that the number of spore cells per  $(10\mu)^3$  was approximately 14.6.

per cent. Undoubtedly some part of this variability lies in the error of measurement.

*The proportions of individuals fruiting at 17°C. in the light.*—The observation that fruiting bodies of *D. discoideum* grown in light show a disproportionately large stalk was first made by Raper (1941). An attempt has been made here to study this effect quantitatively.

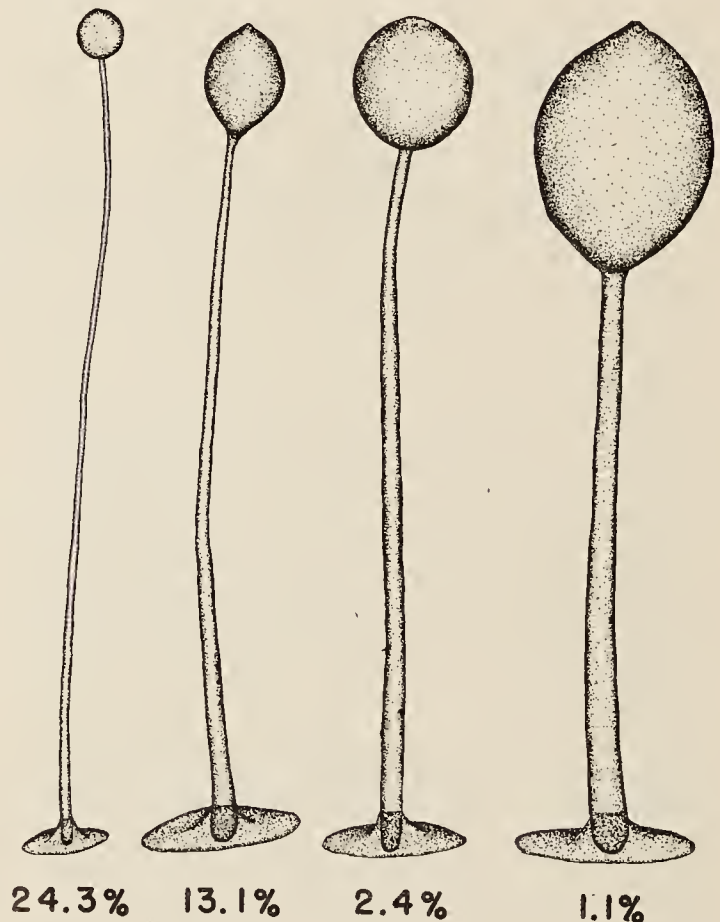


Fig. 2. Camera lucida drawings to show the appearance of fruiting bodies of different proportions. The ones drawn here are of approximately equal height. The percentage of stalk cells is indicated below each sorocarp.

A 25 watt light bulb was placed 16 in. away from the slime mold culture and the two were separated by a piece of Alko glass (Corning Glass Co.) which absorbs the larger wave lengths including the infra red and some of the red.<sup>5</sup> This arrangement was placed in an air circulating incubator set at 17°C. As before, the myxamoebae were grown for 2 days at room temperature (21–24°C., diffuse light) and then placed under the conditions described above.

In a study of thirty-one cases, measured by the segment method, the observation of Raper was completely supported. It was found that 24.0 per cent of the cells were stalk cells (76.0 per cent spore cells) and that the standard deviation was  $\pm 4.50$  per cent stalk cells. (See fig. 2 for the

<sup>5</sup> Some experiments were done without the Alko glass and there was no significant difference in the result. Since in both cases the effect of light is the opposite of the effect of increased temperature it may be assumed that it is a true light effect and not simply a heat effect.

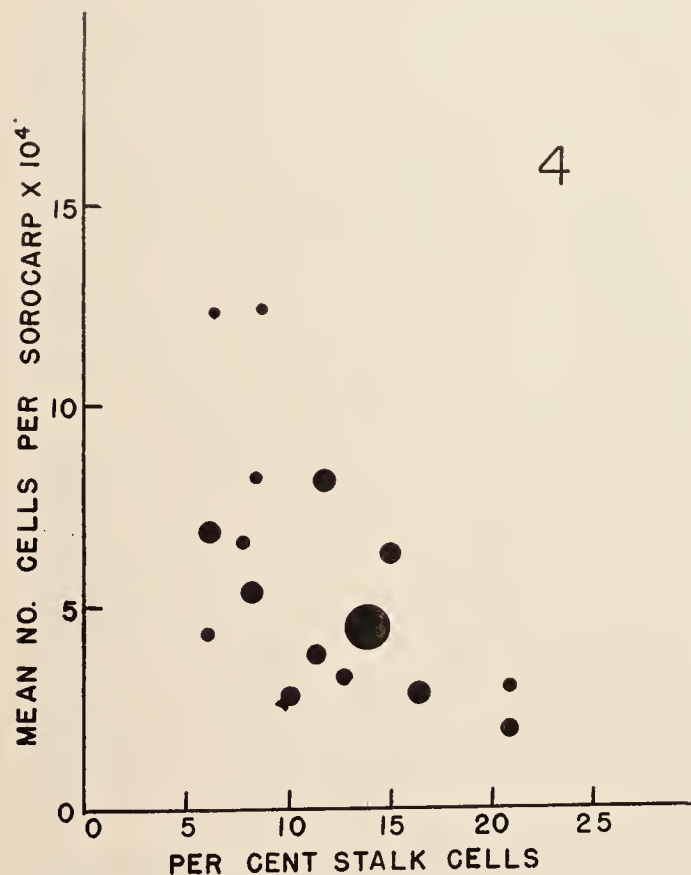
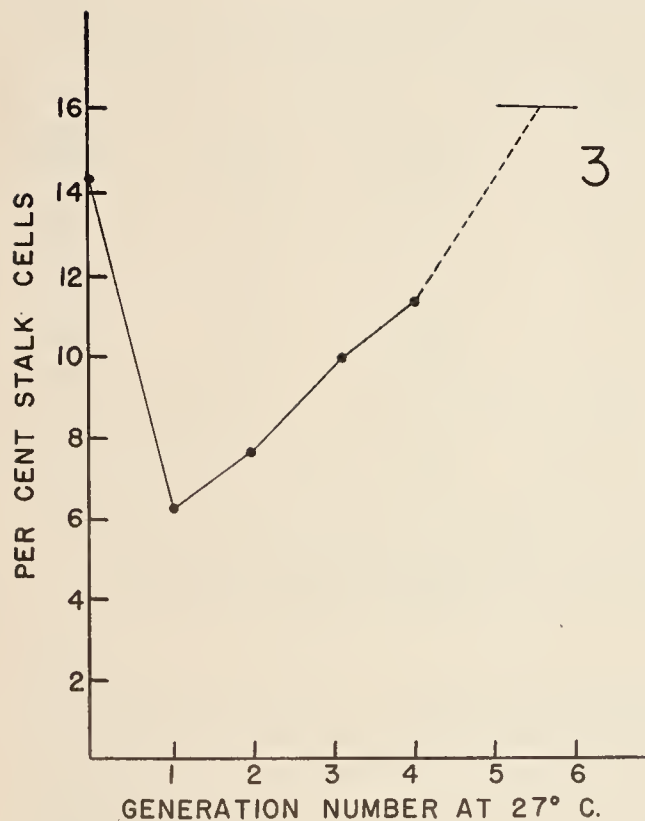


Fig. 3-4.—Fig. 3. Graph showing the percentage of stalk cells of slime molds that have fruited repeatedly at 17°C. in the dark (0 on the abscissa) and then have been subjected to 27°C. in the dark during their fruiting phases for a number of successive generations. *Note:* This curve represents two separate experiments, one of

appearance of sorocarps of different proportions.)

*The proportions of individuals fruiting at 27°C. in the dark.*—The first experiment to determine the effect of temperature was done at 27°C. in the dark. As before the myxamoebae were first grown at room temperature (21–24°C.) for 2 days and when they fruited at 27°C. it was noticed that no migration had occurred and that the stalks appeared very small in proportion to the huge sori. Upon measurement of twenty-two cases by the segment method it was found that an average of only 2.2 per cent of the cells were stalk cells (97.8 per cent spore cells) and the standard deviation was  $\pm 3.39$  per cent stalk cells.

Another interesting fact concerning these highly disproportionate fruiting bodies, is that besides having few stalk cells, the cells themselves were large compared to the other two series discussed. Normally the number of stalk cells per  $(10\mu)^3$  was 4.30 but here the number of cells per  $(10\mu)^3$  was 1.71.

*The change in proportions after fruiting for a number of generations at 27°C. in the dark.*—An experiment was performed to determine whether or not the disproportionately small-stalked condition achieved in the first generation at 27°C. would become altered in successive generations fruiting at that same temperature.

The results of this experiment all plotted in fig. 3 show that after five or six generations the proportions have returned to approximately those obtained at 17°C. (Dark) (*i.e.*, 14 per cent stalk cells).

This fact is especially remarkable when one considers that each generation was, as before, grown at room temperature (21–24°C., diffuse light). In other words all the growth, or at least a large share of it, was at a low temperature (21–24°C.). Therefore, this adaptation to increased temperature must have occurred during the fruiting phase only, making it highly unlikely that any selection by differential growth was involved.

Also, it should be noted that no migration occurred in the early generations, but in the fourth, fifth, and sixth slight but progressively increasing migration was observed.

Finally the 27°C. adapted culture was, in a seventh generation, placed at 17°C. in the dark and it showed abnormally long migration, followed by fruiting bodies having a mean of 15.6 per cent stalk cells (21 cases measured by the Turrell method) which is approximately normal for 17°C. in the dark (*i.e.*, 14.1 per cent stalk cells).

which was taken only as far as the fourth generation (it was then lost by an unfortunate accident) and the other was an early experiment in which only the fifth and sixth generation were measured and they were both measured together.—Fig. 4. Graph showing the relation between the mean sorocarp size of any population and the mean percentage of stalk cells of the population. cases involved.

The size of the dots indicate the relative number of



TABLE 1. Table showing the effect of a change of temperature ( $\Delta T$ ) on the fruiting of *Dictyostelium discoideum*.

$\Delta T$	Temperatures				Mean % stalk cells	Extent of migration	Number of cases
	Previous 5 fruitings	Vegetative stage	Migration stage	Culmination stage			
0	17°	R <sup>a</sup>	17°	17°	14.1	+++	56
	17° (2°) <sup>b</sup>	R	17°	17°	12.9	+++	20
	17°	2°/R <sup>c</sup>	17°	17°	9.2	++	35
	17°	R/2°/R <sup>d</sup>	17°	17°	16.3	+++	30
	27°	R	27°	27°	16.3	++	17
10°	17°	R	27°	27°	2.2	0	37
	17°	27°	27°	27°	2.1	0	20
15°	17°	R	17°/2°/17° <sup>e</sup>	17°	2.9	0	23
25°	17°	R	17°/2°/17° <sup>e</sup>	27°	0.9	0	6

<sup>a</sup> R = Room temperature, i.e., 21–24°C.  
<sup>b</sup> Here, although previous fruiting had been at 17°C., the quiescent spores had been kept for over 5 months at 2°C.  
<sup>c</sup> Five days at 2°C. immediately after inoculation, followed by the standard 2 days at room temperature.  
<sup>d</sup> After inoculation, 1 day at room temperature, 3 days at 2°C., then one more day at room temperature.  
<sup>e</sup> Migrating pseudoplasmodia were put at 2°C. for 2 days and then placed at the given culmination temperature.

*The effect of the change of temperature on the proportions.*—The above experiments suggest that a sudden increase in temperature has a marked effect on the proportions, while a decrease has none. It was possible by some further experiments to obtain more quantitative and specific information concerning the effect of an increase in temperature.

A comparative picture of the effects of no increase in temperature, to an increase of 10°, 15°, and 25°C. can be seen in table 1 and the trend shows that the greater the change in temperature the smaller the percentage of stalk cells.

The increase in temperature has a marked effect only when the change is made during a morphogenetic phase (migration and culmination). If the temperature is depressed during the growth phase only (cases b, c, and d) then the effect of the temperature change is either very slight or non-existent.

Some tests were run to determine how late in the life cycle one could obtain this temperature-increase effect and it was found that beyond the early culmination stage (culm-1 or culm-2, Bonner, 1944) the temperature had no effect on proportions.

*The correlation between the mean per cent stalk cells and the mean size of the fruiting bodies.*—Since for each of the experiments reported in the previous sections, not only was the percentage of stalk cells recorded, but also necessarily the size (or number of cells) of each sorocarp, an attempt was made to determine if the mean size of the sorocarps was a function of the mean percentage of stalk cells (fig. 4). Despite the great scatter of the points in this graph, clearly the greater the percentage of stalk cells, the fewer cells in a mean size sorocarp. The scatter is, no doubt, in part caused by the normal extreme variability of size of any one population. Take as an example an instance where there are 21 cases with a mean size of 18,912 cells, then the standard deviation is  $\pm 12,247$  cells.

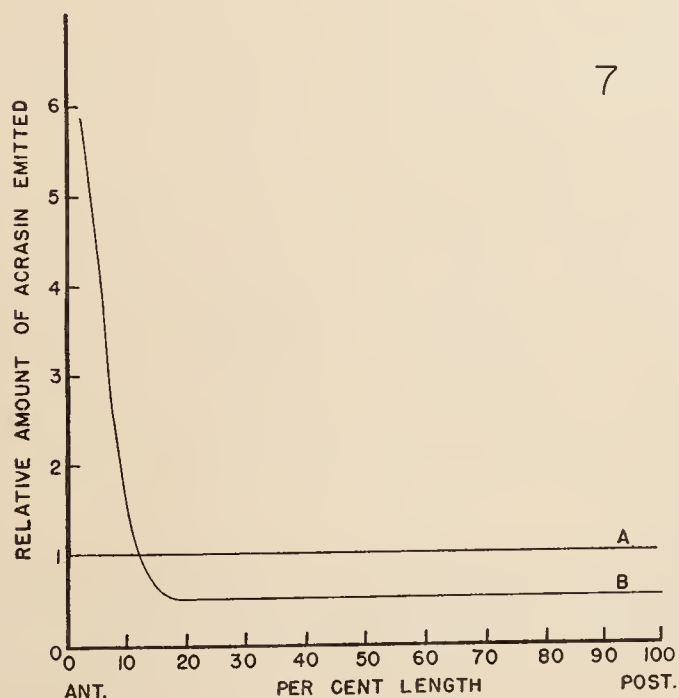
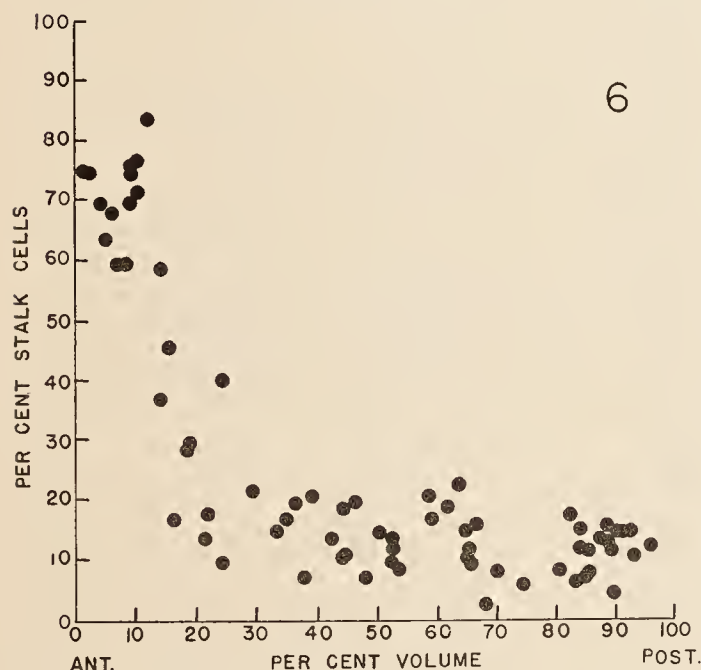
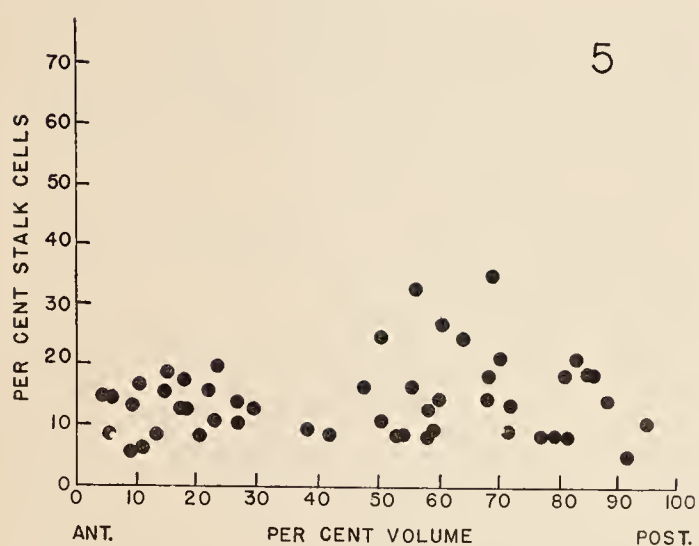
*The proportion of amputated sections of migrating pseudoplasmodia (at 17°C. in the dark).*—It

has been shown by Raper (1940) that if a migrating pseudoplasmodium is cut transversely into sections, the anterior section, provided it fruits immediately without further migration, will have a disproportionately large stalk, while the posterior sections will appear normal in proportion. Again Raper's observations were examined with the quantitative technique developed here.

In the first place it was found that this phenomenon was only true for migrating pseudoplasmodia that had already migrated some distance, and those young ones that had not migrated at all showed no difference between the anterior fraction and the other fractions. (fig. 5). The procedure was to cut the migrating pseudoplasmodium up into either two portions (ten cases) or three portions (ten cases) and after having fruited in the dark at 17°C. their proportions were measured by the Turrell method. The relative size of the fractions was calculated on the basis of percentage of the total volume. Then, in fig. 5, these fractions were placed in their proper antero-posterior sequence along the abscissa. The midpoint of each of the fractions (abscissa) was plotted against the percentage of stalk cells of the fraction (ordinate). In this way the relative position of the percentage of stalk cell producing tendency was indicated along the axis of the migrating pseudoplasmodium. As can be clearly seen from the graph, although the data are variable, the fractions are on the average equal in percentage of stalk cells the length of the pseudoplasmodium, having a mean in the neighborhood of 14 per cent stalk cells, the expected normal value for 17°C. in the dark, as shown before.

In cases where the migrating pseudoplasmodium had migrated some distance (1–4 cm.), the anterior fraction showed a very definite high percentage of stalk cells (fig. 6), but the posterior 75 per cent of the pseudoplasmodia showed an even distribution of proportions, again about 14 per cent stalk cells. (These experiments involve seven cases cut





into three fractions, and twelve cases cut into four fractions.)

Thus, between the very beginning of culmination and a later period there is a marked increase in the number of stalk cells produced by the anterior portion, if fruiting occurs directly after cutting, while there is no change in the stalk-producing ability of the posterior 75 per cent.

*The correlation between acrasin distribution and the distribution of proportions along the axis of the migrating pseudoplasmodium.*—It has been shown previously (Bonner, 1947) that there is good evidence that a substance which has been called acrasin is responsible for the aggregation of the amoebae by chemotaxis. Furthermore, it was found that acrasin continued to be produced in the later stages and it was possible to map out the relative ability of different parts of the pseudoplasmodium at different stages to produce acrasin (Bonner, 1949).

Specifically, it was found that in older migrating pseudoplasmodia (those that had already migrated some distance) the anterior tip produced a large amount of acrasin, while the whole remaining portion produced very little, as shown in fig. 2 of this previous paper (Bonner, 1949). Since the slope of the points in this figure indicate the acrasin producing gradient, it has been redrawn here (fig. 7B) with the slope as the ordinate.<sup>6</sup>

There is an obvious similarity between the acrasin producing ability (fig. 7B) and the proportion of fractions (fig. 6) along the axis of older migrating pseudoplasmodia. If these two phenomena are really correlated, however, one would expect no gradient of acrasin along young pseudoplasmodia that had not migrated at all, since there is an even

<sup>6</sup> This slope can be only approximate. See Bonner (1949) for reasons.

Fig. 5-7.—Fig. 5. Graph showing the distribution of stalk cell producing tendency along the axis of young migrating pseudoplasmodia that have not migrated. A hypothetical example will be used to illustrate the nature of the graph. A pseudoplasmodium was cut into three fractions which were allowed to fruit. The anterior fraction was 20 per cent of the total volume and showed 13.2 per cent stalk cells upon fruiting. The first point on the graph for this pseudoplasmodium would then be 13.2 per cent stalk cells on the ordinate and 10 per cent volume on the abscissa, i.e., the mid-point of the fraction. If the middle piece was also 20 per cent of the total volume then its mid-point would be at 30 per cent on the abscissa. Likewise the remaining posterior 60 per cent would be represented by a dot at 70 per cent volume and the appropriate percentage of stalk cells.—Fig. 6. The same as fig. 5 except for older migrating pseudoplasmodia that have migrated in the neighborhood of 1-4 cm.—Fig. 7. A. The acrasin emission along the axis of young migrating pseudoplasmodia (see text for explanation of method). B. The relative acrasin emission along older migrating pseudoplasmodia. This curve represents the slope of fig. 2 in a previous paper (Bonner, 1949). This curve is not accurate and is only a diagrammatic approximation. See Bonner (1949) for the reasons of its limitations in accuracy.

distribution of proportions given by the fruiting of the various fractions (fig. 5).

It was found by experiment that such is precisely the case. In five tests, using the technique of cutting the pseudoplasmodia in two and testing the strength of the portions by their ability to attract myxamoebae (Bonner, 1949), it was consistently demonstrated that there is no gradient of acrasin producing ability along the axis of young migrating pseudoplasmodia as shown in fig. 7A.

DISCUSSION.—In order to fully understand these results the reader must be reminded that from the moment the cells of *Dictyostelium* come together to form a pseudoplasmodium there is no cell division, no growth. In some way the sausage-shaped migrating pseudoplasmodium is divided into anterior pre-stalk cells and posterior pre-spore cells, and this division takes place without morphogenetic movements (Bonner, 1944).

We have shown that there is a good direct correlation between the ability of parts of the pseudoplasmodium to produce acrasin and their tendency to form stalk cells. In intact pseudoplasmodia grown under conditions which produce normal migration, the anterior end of the pseudoplasmodium after a period of migration produces the large share of acrasin, and contributes the stalk cells to the final sorocarp. If the temperature is suddenly increased, then there is no migration, therefore, presumably no high emission of acrasin at the anterior end which correlates with a very low percentage of stalk cells in the resulting fruiting body. Also, in the experiment which involved the sectioning of the migrating pseudoplasmodium into fractions, the correlation between acrasin production and percentage of stalk cells was consistent (fig. 5, 6).

The fact that the mean size of any population of fruiting bodies is very approximately inversely proportional to the proportions (*i.e.*, percentage of stalk cells, fig. 4) may possibly also be explained by the above correlation between acrasin and proportions. The number of cells that enter a pseudoplasmodium, and hence its size, must presumably be dependent on the amount of acrasin emitted from each cell during aggregation. Stronger forces of mutual attraction, all other things being equal, can be shown to favor smaller clumps when a mutually attracting system of particles changes from a nearly uniform distribution to clumps because of random motions. (For physical analogy see Jeans, 1928, formula 316.2.)<sup>7</sup> Thus assuming such be the case in *Dictyostelium*, then more acrasin per cell would produce more numerous small pseudoplasmodia, in which, since the acrasin is high per cell, the proportions will show, as is the case, a high percentage of stalk cells.

Such a correlation between the percentage of stalk cells and the acrasin production still does not

tell us of the causative sequence of events. And even were it known that acrasin directly affected the percentage of stalk cells, the whole problem of the reasons for the particular distribution of acrasin in the pseudoplasmodium, which is intimately tied up with the problem of its polarity, would still remain unexplained. Also it is not entirely clear at present why in any one environmental condition the proportions are the same irrespective of the size of the fruiting body.

We can, however, make a few remarks which have a bearing on the subject of the process of regulation. Fortunately, with the use of a sudden change in temperature as a tool it is possible to upset proportions which will only return to normal, that is regulate, after a period at constant temperature. It was shown that this temperature change would only have an effect if it was an increase in temperature, and if it was applied at any time during migration up to early culmination (the aggregation stage was not tested). It is assumed that the effect will not take place in late culmination because the differentiation has become irreversible or determined.

Apparently regulation back to normal proportions takes time and it can only be time expended during the morphogenetic phases of the life cycle (presumably aggregation and certainly migration). The evidence for regulation not taking place during the growth phase is that in the culture which was placed at 27°C. throughout the whole life cycle there was no regulation and no migration, (table 1). It is possible that a decrease in temperature has no effect simply because migration is extensive and there is ample time for regulation.

Ultimately it may well prove to be significant that it is only during the morphogenetic phases (which we have just said are the only stages where proportions can be altered and regulation take place) that acrasin is secreted. Furthermore, it is known that the vegetative myxamoebae do not even respond to acrasin chemotactically, and will only do so when they enter the aggregation stage where they become simultaneously capable of emitting and responding to acrasin (Bonner, 1947).

That regulation can only take place during the morphogenetic phases is an interesting enough fact, but the truly curious phenomenon is that regulation need not take place in one fruiting, but can occur cumulatively over a series of consecutive fruitings, each separated by a period of growth. That is, the extent of regulation, the proportions, are accurately recorded in the spores, this information sometimes preserved there for months of dormancy and more than that it is preserved through a growth phase, a period of many rapid successive cell divisions.

Since the proportions are correlated with the ability to produce acrasin, it is always possible that what is retained by the spore and what is retained through the growth phase, is a set limit of acrasin producing ability, which would affect

<sup>7</sup> The authors are very indebted to Dr. John W. Tukey of the Department of Mathematics, Princeton University, for this reference and also for assistance on the statistics in this paper.



the mean size of the population and their proportions. But still we do not seem to have quite grasped the mystery of regulation, for there are still many aspects of any scheme based on these data that must be tested by further experiments.

#### SUMMARY

In the amoeboid slime mold *Dictyostelium discoideum* growth and cell division occur first and are followed by the differentiation process. Therefore, in this study of the proportions of spore cells and stalk cells growth is not concerned, but solely differentiation. The following are the principal facts concerning proportions that were obtained. The proportions of spore and stalk cells were found to be constant irrespective of the size of the pseudoplasmodium in any one environmental condition. Sudden increases in temperature would reduce the

percentage of stalk cells, but after repeated generations at the new temperature the proportions would return to a norm. Temperature had this effect only during a fruiting phase, and the regulation back to normal proportions also took place only during a fruiting phase. The proportions of one generation could affect the proportions of the following generation indicating that a proportions-determining mechanism is retained in the cells through a quiescent spore stage and through a growth stage of numerous successive cell divisions. A correlation was found between the ability of certain regions to produce a large amount of acrasin, and the tendency of those regions to have a high percentage of stalk cells.

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## THE DEMONSTRATION OF ACRASIN IN THE LATER STAGES OF THE DEVELOPMENT OF THE SLIME MOLD *Dictyostelium discoideum*

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THREE FIGURES

### INTRODUCTION

The amoeboid slime mold *Dictyostelium discoideum* is well suited for the study of problems of development. In its simple life cycle the processes of growth and cell multiplication are normally separated from the purely formative, morphogenetic processes. A capsule-shaped spore germinates and liberates a myxamoeba which when fed with suitable bacteria produces by successive binary fissions a mass of separate, independent myxamoebae. After a period this vegetative stage abruptly stops and the cells stream into central collection points (aggregation stage) and this transition is one from a growth phase to a morphogenetic phase. The cell mass, or pseudoplasmodium, resulting from aggregation becomes elongate and moves as a cartridge-shaped body over the substratum for variable periods of time. When its forward motion ceases it turns upright into the air (culmination stage) and simultaneously differentiates into a thin, tapering stalk, a rounded apical spore mass or sorus, and a basal disc surrounding the base of the stalk. (See Raper, '35, '40a, '40b, '41, and Bonner, '44, for descriptive details.)

Good evidence has been given previously (Bonner, '47) to show that during aggregation the myxamoebae are guided by chemotaxis towards the central mass, and the active, re-

sponsible substance has been tentatively called *acrasin*. As was pointed out then, acrasin, by virtue of its existing in a diffusion gradient, orients the myxamoebae by differential stimulation, but does not necessarily affect the rate of the myxamoeba locomotion.

It is obvious, from the fact that the myxamoebae form streams and do not make a direct beeline to the center, that not only does the center produce acrasin, but also the streams to a lesser extent.<sup>1</sup>

The purpose of this present paper is to determine if acrasin exists in the later stages of development, migration and culmination, and if so to map out the relative ability of various regions to produce acrasin.

#### RESULTS<sup>2</sup>

The following method was used to determine the presence or absence of acrasin in the migrating and culminating pseudoplasmodia. A Syracuse dish containing numerous myxamoebae (in standard solution) which were about to aggregate was covered with a layer of mineral oil (over the standard solution). A migrating pseudoplasmodium removed with a platinum needle from an agar culture and placed in the dish, adhered to the mineral oil interface.<sup>3</sup> With a pipette most of the water was removed from under the oil so that the oil-water interface was very near the water-glass interface. In a very short period (3 to 5 minutes) it could be observed that the separate myxamoebae on the bottom of the dish became oriented and began to move toward the migrating pseudoplasmodium. This was found also to occur with a culminating pseudoplasmodium.

<sup>1</sup> It is not known if this is because each myxamoeba produces the same amount of acrasin and there are simply more myxamoebae at the center, or that for some reason the individual myxamoebae at the center produce relatively more acrasin.

<sup>2</sup> The basic methods employed here are the same as those described previously (Bonner, '47).

<sup>3</sup> It was known, from the work of Potts ('02) that *Dictyostelium* will go through its normal development under a layer of mineral oil.



Since the presence of acrasin is clearly demonstrated in these later developmental stages, it was decided to determine the distribution of acrasin production in the various regions of the migrating and culminating pseudoplasmodia. This mapping of the acrasin production was done in two ways, only one of which is applicable to the culmination stage.

TABLE 1

*Data from the experiment in which a migrating pseudoplasmodium was cut transversely into two pieces, an anterior one (A) and a posterior one (P). The original total length of the pseudoplasmodium ( $L_A + L_P$ ) is given in  $\mu$  in column 2. The per cent length of the anterior portion ( $\%L_A$ ) is given in column 3. The pieces were placed a certain distance apart ( $R_A + R_P$ ) as indicated in column 4. A division line appeared in the random myxamoebae between those that went to the anterior portion and those that went to the posterior portion. The per cent distance between the anterior portion and the division line ( $\%R_A$ ) is given in column 5*

1	2	3	4	5
EXPERIMENT NUMBER	TOTAL LENGTH ( $L_A + L_P$ ) in $\mu$	$\% L_A$	$R_A + R_P$	$\% R_A$
224	1448	38	1520	69
230	740	42	1233	64
236	442	43	742	64
247	1160	11	1190	62
248	1200	6	513	56
250	608	33	614	60
251	832	56	775	74
253	593	18	654	64
255	1580	18	810	48
256	715	22	560	59
257	772	7	675	54
258	696	31	900	56
260	802	25	440	63
262	574	31	506	56
264	775	61	762	66
265	965	57	858	77
269	472	53	735	83
279	729	30	1110	73
280	708	11	1140	44
292	648	14	736	60
294	972	5	614	21
295	408	17	614	59
296	398	34	614	52
331	1120	10	1060	45

The first method consisted of removing a migrating pseudoplasmodium from an agar culture dish and with fine hair needles pushing it below the surface of some standard solution in a Syracuse dish, so that it rested on the bottom in the vicinity of myxamoebae that are about to aggregate.<sup>4</sup> Then, with either a hair loop or a fine glass needle, the pseudoplasmodium was cut transversely. The two pieces were then placed a short distance from one another making sure that a good field of random myxamoebae about to aggregate lay between them. The two pieces would then proceed to compete in an attempt to attract the intervening myxamoebae. In 10 to 20 minutes it is possible to see a clear division line in the aggregating myxamoebae between those that go to one piece of the migrating pseudoplasmodium and those that go to the other (see fig. 1, A).

In each case two measurements were made from camera lucida drawings. One measurement was of the length of the pieces involved. This actually was done by first measuring the intact pseudoplasmodium and the length of the cut portions so as to check on any shrinkage or loss that might be caused by cutting. In most cases this precaution was found to have been unnecessary. The other measurement was the distance from the estimated center of attraction of the pieces to the division line of the aggregation myxamoebae. (See table 1.) Assuming that our original postulate, that aggregation is governed by a diffusion gradient is correct, then at the division line the concentration of acrasin moving from the anterior portion must equal that coming from the posterior portion. Therefore, the relative distance between the anterior portion and the division line and the posterior portion and the division line are an index of the relative amounts of acrasin produced by the two pieces.

To compare two pseudoplasmodia of different size the absolute lengths have been converted to the per cent lengths of the anterior portion. Also the distance between the anterior

<sup>4</sup>See Bonner ('47) for the method of preparation of the aggregating myxamoebae.

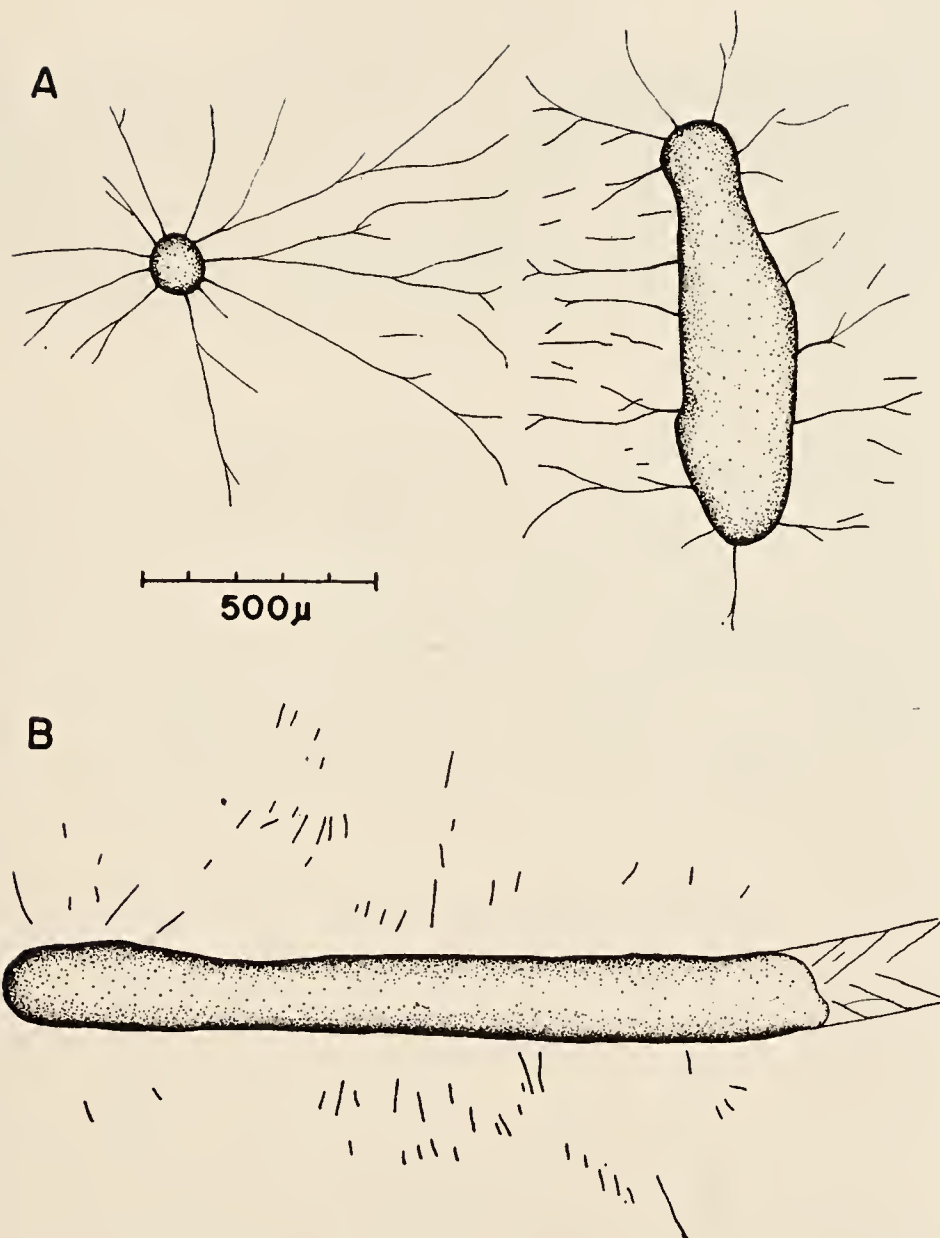


Fig. 1 Camera lucida drawings illustrating the two types of experiment used to determine the distribution of acrasin production in the migrating pseudoplasmodium. *A*. A small anterior portion (left) and a large posterior portion (right) of a migrating pseudoplasmodium are competing for aggregating myxamoebae in an under water preparation. Note the division line between those myxamoebae that are going to the anterior portion and those that are going to the posterior portion. *B*. An intact migrating pseudoplasmodium which has been placed on a thin film of water containing aggregating myxamoebae. It is possible to obtain the relative distribution of acrasin production by drawing contour lines perpendicularly through the oriented aggregating myxamoebae (see fig. 3).



portion and the division line has been converted to a per cent of the total distance between the two pieces (table 1). These two relationships have been plotted on a graph (fig. 2). Some further experiments were done in which the migrating pseudoplasmodia were cut into three pieces, then an average value

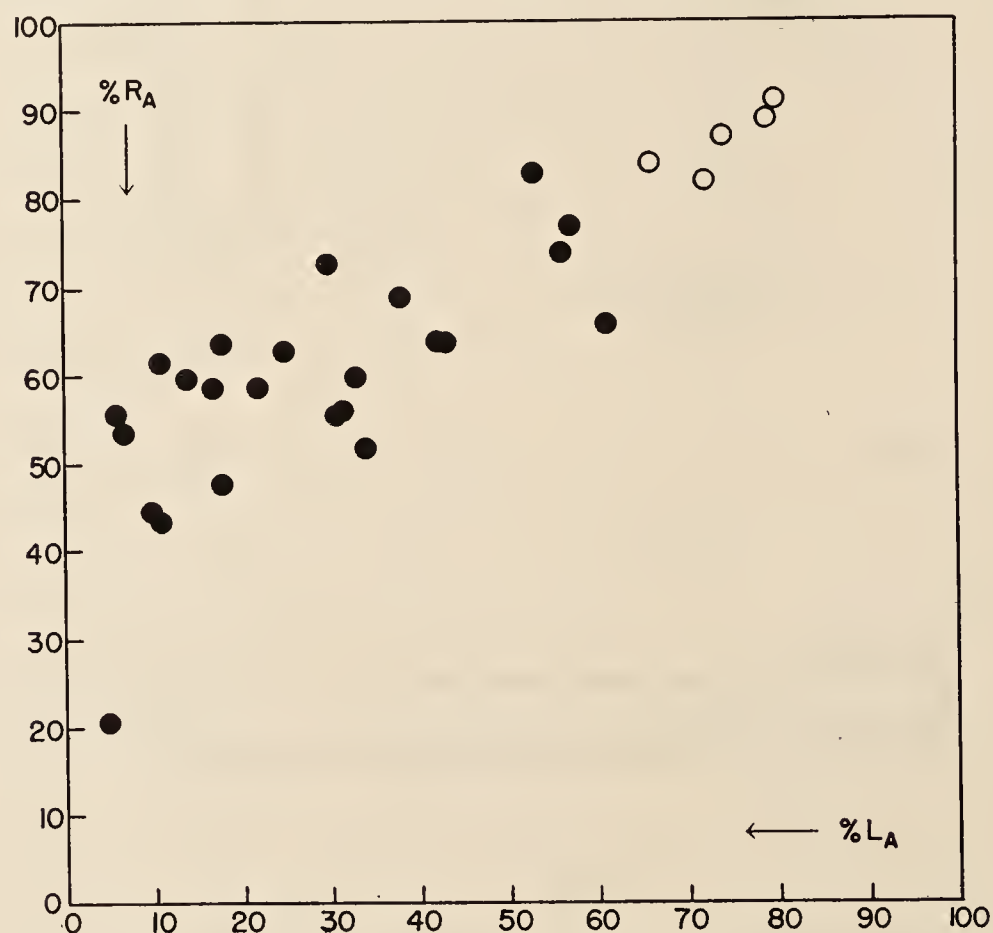


Fig. 2 A migrating pseudoplasmodium was cut transversely and the anterior portions were made to compete for aggregating myxamoebae with the posterior portions. A sharp division line formed between those amoebae that went to one portion and those that went to the other. The ordinate of the above graph is the distance between the anterior portion and the division line expressed in per cent of the total distance between the two pieces ( $\% R_A$ ). The abscissa is the per cent length of the anterior portion ( $\% L_A$ ). The solid dots indicate those experiments in which the pseudoplasmodium was cut into two portions (table 1). The circles indicate those experiments in which the pseudoplasmodium was cut into three portions and an assumed value was given for the anterior portion, testing only the middle and posterior portions (table 2).

was assumed for the distance (or strength) of the anterior portion, and a test was made of the middle and posterior portions (see table 2). These are indicated in figure 2 by circles.

It is hard to obtain much precise information from this graph, but one fact does emerge: namely that about 50% of the total amount of acrasin produced is produced in the apical

TABLE 2

*Data from the experiment in which a migrating pseudoplasmodium was cut transversely into three pieces: an anterior (A), middle (M), and a posterior (P) piece. The original total length of the pseudoplasmodium is indicated in column 1. The percent length of the anterior (%  $L_A$ ), middle (%  $L_M$ ) and posterior (%  $L_P$ ) portion is indicated in column 3, 4, and 5, respectively. The middle and the posterior portions were selected and placed a certain distance apart ( $R_M + R_P$ ) as indicated in column 6. A division line appeared in the random myxamoebae between those that went to the middle portion, and those that went to the posterior portion. By placing the value for the %  $L_A$  on the graph in figure 3 it is possible to obtain an expected or assumed value for %  $R_A$  which is indicated in column 7. Knowing the %  $R_A$  and the actual distances in  $\mu$  of  $R_M$  and  $R_P$ , the %  $R_M$  and %  $R_P$  follow and are given in columns 8 and 9, respectively*

1	2	3	4	5	6	7	8	9
EXPERI- MENT NUMBER	TOTAL LENGTH IN $\mu$	% $L_A$	% $L_M$	% $L_P$	$R_M + R_P$ in $\mu$	ASSUMED % $R_A$	% $R_M$	% $R_P$
281	702	17	49	34	1190	57	27	16
287	925	34	38	28	627	66	16	18
288	930	31	43	26	775	64	23	13
289	1090	49	30	21	573	74	15	11
290	695	62	17	21	492	81	10	9

1/10 of the migrating pseudoplasmodium. The remaining 50% of the acrasin producing ability is distributed over the posterior 9/10, and it is impossible to know precisely how much the various parts within this 9/10 produce acrasin, although they all must do it to some extent.

Attempts were made to determine whether or not there is a correlation between the variation and such a factor as absolute size, but none was found.

The one great difficulty with these experiments is that one is introducing the unknown effects of cutting. To what extent does the major operation affect the acrasin producing ability? Another difficulty was that the underwater environment is abnormal. A few experiments of the same type were made on a hanging drop preparation using a thin film of water and having the pseudoplasmodium in the normal milieu of moist air. The results were identical with the underwater experiments.

The second type of experiment which was used to determine the regional differences in acrasin production of pseudoplasmodia has the disadvantage of being less quantitative, but the advantage of leaving the pseudoplasmodium untouched and in an entirely normal condition. It consists of placing a pseudoplasmodium in the vicinity of myxamoebae that are about to aggregate and noting the *direction of orientation and movement* of all the cells in its immediate neighborhood (see fig. 1, B). It was found helpful to place directly on the camera lucida drawings contour lines drawn orthogonally through the incoming myxamoebae, and in this way the general distribution of acrasin gradients, and hence zones of high and low production could be obtained.

The technique briefly was as follows: Pseudoplasmodia were placed, under moist conditions, on the surface of standard solution in a Syracuse dish. Myxamoebae which were beginning to aggregate were on a coverslip on the bottom of the dish. This coverslip was lifted up and out of the Syracuse dish so that the pseudoplasmodium rested in its center and the excess water was then allowed to drain off. Still under moist conditions, this coverslip was placed on a van Tieghem cell and the preparation placed under the microscope.

A number of facts (which are illustrated in figure 3) were brought to light by this method. (1) The anterior tip showed the greatest amount of attraction, agreeing with the results obtained by the previous cutting method. (2) In elongate migrating pseudoplasmodia, the posterior 9/10 usually showed





Fig. 3 Diagram illustrating the distribution of acrasin production in migrating and culminating pseudoplasmodia. *A*, Frequently observed distribution of acrasin production in an elongate migrating pseudoplasmodium. *B*, *C*, Successive stages of shortening of the migrating pseudoplasmodium showing the accompanying appearance of a steep acrasin production gradient. *D*, *E*, Early and late culmination showing that acrasin is produced and emitted by the tip only.

a fairly evenly distributed ability to attract aggregating myxamoebae (i.e., the myxamoebae were always perpendicular to the main axis of the migrating pseudoplasmodium). (3) Occasionally there would be a secondary active region somewhere in the posterior portion, but usually an even production of acrasin was indicated. (4) If this elongate pseudoplasmodium contracted to a short, thick form, to enter the culmination stage (or merely did so as a momentary response to adverse conditions and later became elongate again) then the orientation of myxamoebae indicated a steep gradient of acrasin producing ability, highest at the anterior tip, and lowest at the posterior end. Thus a change from an elongated to a contracted form of the migrating pseudoplasmodium, has been accompanied in all cases observed, by a change in the acrasin producing pattern from a moderately high tip region and a fairly evenly producing main body, to a steeply graded pattern. (5) In the culminating pseudoplasmodium the only region that emits acrasin at all is the very tip<sup>5</sup> and occasionally to a slight degree the pre-stalk cells, again in a graded fashion. The pre-spore or spore cells or final stalk cells do not produce acrasin at all.

Thus there is evidence that in aggregation acrasin is produced by all the parts, and that this is also true for the migration stage, but in the transition from migration to culmination, the ability to produce or at least emit acrasin is progressively lost in the posterior regions of the pseudoplasmodium. Furthermore there is an apparent correlation between the distribution of the acrasin production and the overall shape of the pseudoplasmodium.

#### DISCUSSION

The main question that these experiments raise is the role of acrasin in the migrating and culminating pseudoplasmo-

<sup>5</sup> It is interesting to note that the group of cells that comprise the active acrasin producing tip are never the same, but are constantly being converted into non-acrasin producing stalk cells.

dia. As was previously shown (Bonner, '47) there is strong evidence that during the aggregation stage the actively pseudopodial myxamoebae are guided inward by the differential stimulus of an acrasin gradient. It is quite possible that acrasin fulfills the same function, that of guiding the myxamoebae chemotactically, in migration and culmination.<sup>6</sup> There is no direct evidence for such a hypothesis, but merely a correlation between the distribution of acrasin production gradients and the shape of the pseudoplasmodium.

Whether or not chemotaxis does control the morphogenetic movement of the later stages, it is clear that other factors do play some part in coordinating the myxamoebae and shaping them into a unitary fruiting body. For instance there are many evidences<sup>7</sup> for the cohesion between cells and closely integrated and often inseparable from this is the action of surface or membrane tension exhibited especially by the slime sheath.<sup>8</sup> Also there are definite indications, which will be reported in a later paper, that the individual myxamoebae have a polarity of their own and that this directly affects their direction of movement.

There is no question, as Raper ('40b, '41) has already pointed out, that the apical 1/10 of the pseudoplasmodium has

<sup>6</sup> It is known that the myxamoebae during migration and culmination are actively pseudopodial up to the moment of differentiation into stalk and spore cells. This has been determined by direct observation with the high powers of a microscope on pseudoplasmodia crawling on the underside of coverslips. Also it is clearly visible in some time lapse motion pictures of a culminating pseudoplasmodium taken through a phase microscope. (Bausch and Lomb Model.)

<sup>7</sup> E.g., in aggregation the cohesiveness of the cell can be reduced by omitting the electrolytes from the medium and then solid streams are no longer formed, but diffuse net-like streams (Bonner, '47).

<sup>8</sup> The slime sheath of the migrating pseudoplasmodium does affect the form of the pseudoplasmodium by membrane tension, for if a fine glass needle is passed back and forth through it the form of such a disrupted pseudoplasmodium is like a flattened drop, and becomes cylindrical again only after a recovery period. Also in culmination the spore mass is no doubt held together by cohesion-tension forces and its very shape is consistent with the principle of minimal areas.



special and unique properties. Raper made the distinction between two functions of the tip, that of receiving and responding by becoming oriented towards such stimuli as light and heat (his "receptive center") and the ability to lead the remaining posterior portion of the migrating pseudoplasmodium (his "directive center"). It has been shown here that this apical region is always one of highest acrasin production and now our task is to ascertain precisely what part acrasin plays in these directing and receiving processes. Lastly there is the extremely important question of the relation of acrasin to the process of differentiation, if any, but unfortunately to this vital problem there are no clues at all.

#### SUMMARY

Evidence was obtained previously to support the view that the first fruiting stage (aggregation) of the amoeboid slime mold *Dictyostelium discoideum* which involves the streaming together of the separate myxamoebae, is controlled by a chemotactically active substance tentatively called *acrasin*.

The following new information has been obtained:

1. Acrasin continues to be produced by the aggregated cell mass throughout its later development (migration and culmination).
2. All the various regions of the migrating pseudoplasmodium produce acrasin (as is the case in aggregation) but the anterior region is especially active.
3. In the transition from migration to culmination the ability to emit acrasin is progressively lost in the posterior portion so that the acrasin is produced at the tip only in the culminating pseudoplasmodium.
4. These changes in acrasin production are correlated with definite changes in the shape of the cell mass, i.e., an even distribution of acrasin production is correlated with an elongate cell mass, and a steep gradient of acrasin production is correlated with a contraction of the cell mass.

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THE ORIENTATION TO LIGHT AND  
THE EXTREMELY SENSITIVE ORIENTATION TO  
TEMPERATURE GRADIENTS IN THE  
SLIME MOLD *Dictyostelium*  
*discoideum*<sup>1</sup>

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THREE FIGURES

The amoeboid slime mold *Dictyostelium discoideum* is unique among the Acrasiales (as Raper, '35, showed when he first discovered it) in that after the independent myxamoebae come together the resulting cell mass, or migrating pseudoplasmodium will crawl about as a unit before it rises into the air and differentiates into an apical spore mass supported by a delicate stalk. It was also noted by Raper ('40 and other papers) that the migrating pseudoplasmodia would move towards light, and in one experiment, in which a petri dish was placed in a heat gradient of approximately 5°C. between the two ends, he demonstrated movement towards the warm end. The original intent of our study was to determine the limits of the action spectrum of the light response, but it soon became evident, as the results will show, that the heat response had to be fully known first.

ORIENTATION IN A TEMPERATURE GRADIENT

The culture medium was the same as that used previously<sup>2</sup> (Bonner, '47). The plates were streaked with *D. discoideum*

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<sup>2</sup> The buffers are incorrectly stated in Bonner ('47). The potassium salt should be  $\text{KH}_2\text{PO}_4$ , and not the dipotassium salt.



spores and *Escherichia coli* and were incubated for two days at 24°C. at which time the myxamoebae had usually begun to aggregate. Circular plugs of the nutrient agar 1.5 cm in diameter containing the most advanced aggregations were then cut out and placed on 2% Bacto agar containing no nutrient. The pseudoplasmodia migrated for an especially long time on plain agar which was a great advantage in these experiments.<sup>3</sup>

The rate of movement of the migrating pseudoplasmodia is relatively slow—in the neighborhood of 2 mm per hour, and for that reason it was found best to run any one experiment at least 12 hours before scoring.<sup>4</sup>

A number of methods were used for obtaining heat gradients, but the most successful and simplest involved heating the under side of an aluminum sheet (39 × 26 × 0.1 cm) with a 7½ watt incandescant bulb covered by black tape and placed in a metal box so that no light could escape. The current through the lamp could be adjusted by means of a variac transformer. Six round wells were formed on the metal surface by pouring paraffin around petri plates and then removing the plates. These wells served to hold the 2% agar and they could be covered with the tops of petri dishes. The heat gradient in the agar was measured with two copper-advance thermocouples (75 mm apart) connected to a galvanometer which indicated the difference in temperature between the junctions. The metal plate was not insulated but exposed in the dark room atmosphere which was around 24°C.

In these heat experiments the orientation was scored by drawing a line through the center of the agar disc, perpendicular to the direction of the heat gradient, and the per cent of the pseudoplasmodia on the warm half was calculated.

If the heat gradient is of sufficient magnitude there is a definite orientation towards the warm region (see fig. 1, B).

<sup>3</sup> A study is being made at the moment of factors affecting the duration of migration.

<sup>4</sup> The rate of migration has been checked at two temperatures. In three cases at 17°C. the mean rate was 1.71 mm/hr and in two cases at 25° the rate was 2.62 mm/hr.

It is possible by reducing the heat gradient to find a point at which there is no orientation (fig. 1, A). From all the experiments performed (table 1) one may plot a graph having the heat gradient per centimeter as an ordinate, and the per cent

TABLE 1  
*Orientation in heat gradients*  
These figures are shown graphically in figure 1, C

HEAT GRADIENT	PER CENT OF PSEUDOPLASMODIA IN THE WARMER HALF					NUMBERS OF PSEUDOPLASMODIA IN EACH EXPERIMENT
$^{\circ}\text{C./cm}$						
.009	63	51	56	47		16,37,18,17
.019	56	41	52	57	54	9,22,25,14,28
.028	58	61	69	72		31,18,13,18
.037	91	73	79	82	77	33,33,14,11,13
.046	57					14
.056	77	83	77	82		13,12,13,44
.066	100					23
.075	82	92				28,12
.084	87	87				8,23
.093	89	91				9,14
.112	81					11
.133	80	100				11,15
.187	90					8
.335	93					29

orientation as the abscissa (fig. 1, C). The remarkable aspect of these experiments is that the migrating pseudoplasmodia are so extremely sensitive to temperature gradients, and that in a gradient of so little as  $0.05^{\circ}\text{C./cm}$  there is decided orientation towards the warmer regions (fig. 1, C).

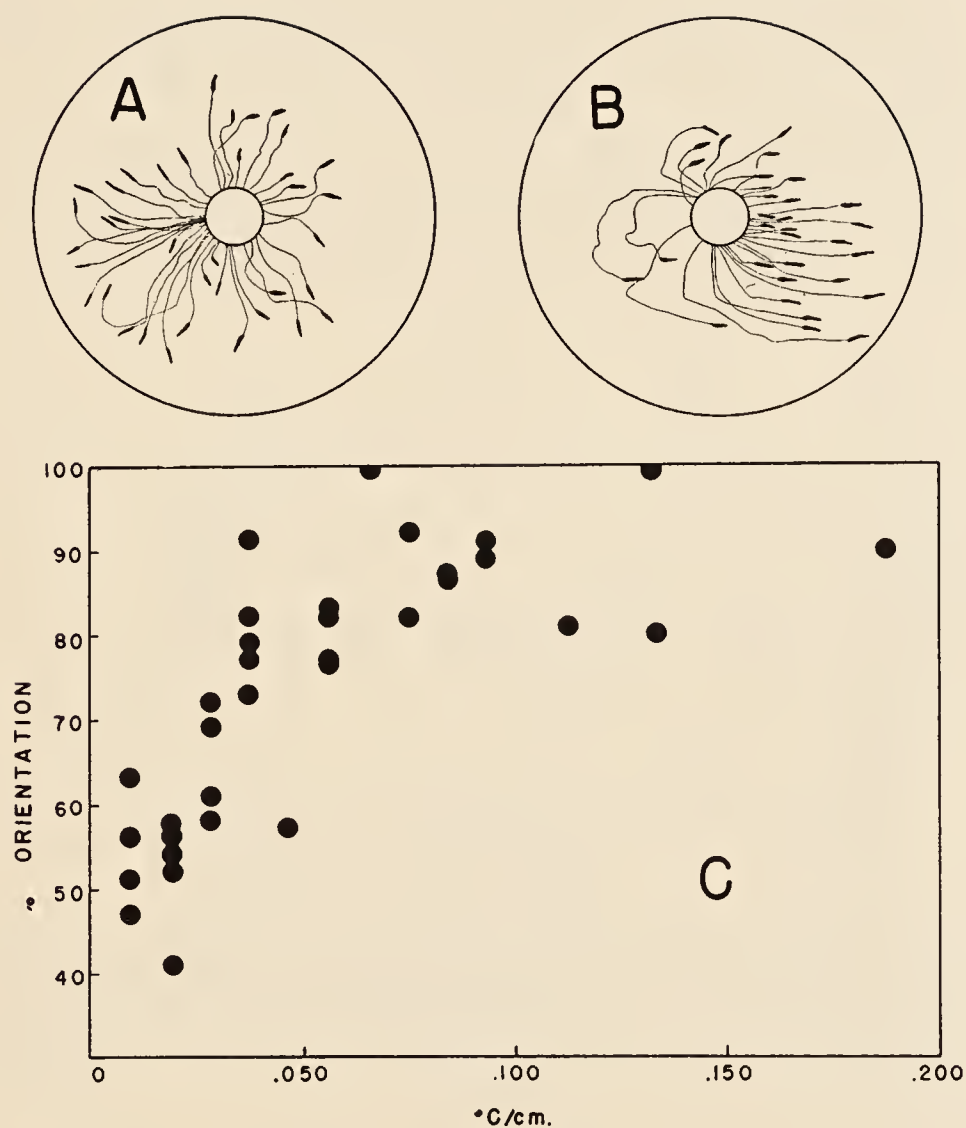


Fig. 1 Orientation in heat gradients. A. A tracing showing the distribution of the pseudoplasmodia and their slime tracks. In this case the heat gradient was  $0.009^{\circ}\text{C./cm}$  and 51% of the pseudoplasmodia are in the warmer half. The source of heat for both A and B is on the right hand side). B. The same in a  $0.037^{\circ}\text{C./cm}$  heat gradient showing 91% of the pseudoplasmodia in the warmer half. C. Graph of the data given in table 1. The ordinate represents the per cent of the pseudoplasmodia in the warmer half, and the abscissa represents the heat gradient in  $^{\circ}\text{C./cm}$ .



## ORIENTATION TO LIGHT

The methods employed here for the culturing and handling of the slime mold were the same as those given before. The 2% agar was placed in the bottom of crystallizing dishes  $90 \times 50$  mm, and each of these dishes was lined on the inside with dull black paper leaving a vertical slit 1 cm wide uncovered in front. The covers and bottoms of the dishes were also blackened so as to be light tight.<sup>5</sup> The experiments were conducted

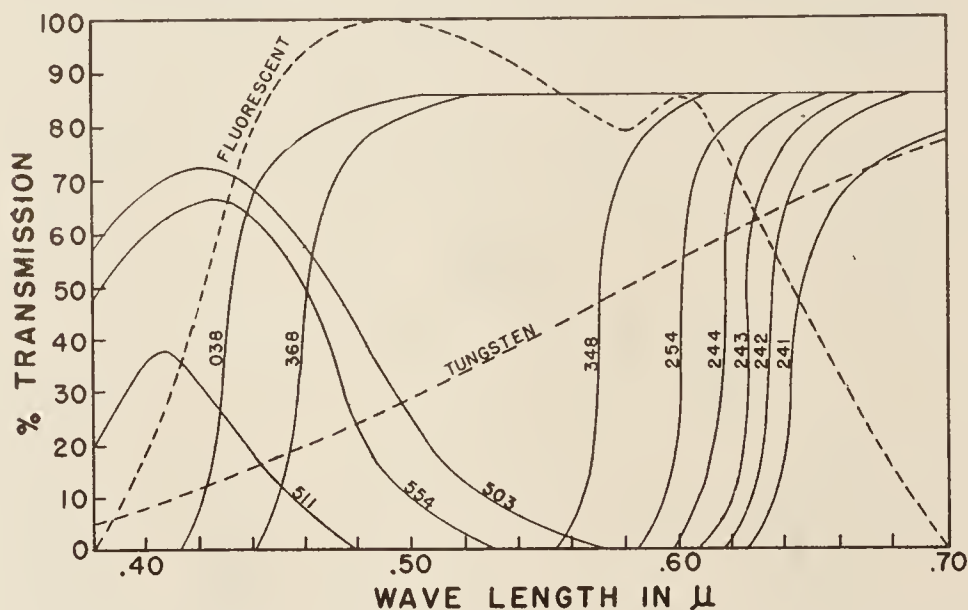


Fig. 2 The absorption spectra of the filters used as given by the Corning Glass Company. The emission spectra of the light sources are given in broken lines. The ordinate of this graph has no meaning for the emission spectra. The line labeled "tungsten" is actually the emission spectrum from a black body at 3000° K and it is included here only to show the general type of emission spectrum obtained from the tungsten lamp.

in a dark room at about 24°C. Two separate light sources were employed; a 15 watt "fluorescent" lamp ("daylight" type), and a 7½ watt tungsten filament lamp. These lamps were kept 5 feet away from the crystallizing dishes which did not give

<sup>5</sup> Different kinds of containers, including rectangular wooden boxes ( $9 \times 12 \times 18$  cm) were used with the same results. Also *Avena* seedlings were occasionally germinated in the vessels and their behavior served as a check against possible light leaks.

TABLE 2  
*Orientation to light*

CORNING FILTER NUMBER	LIMITING WAVE LENGTHS IN $\mu$	ANGLES OF ORIENTATION			NO. OF PSEUDOPLASMODIA INVOLVED IN EACH EXPERIMENT	
		15 watt "fluorescent" (daylight) lamp	7 $\frac{1}{2}$ watt tungsten filament lamp		"Fluorescent"	"Tungsten"
none	(pyrex)	20° 10° 10°	20° 10° 10°		32,38,34	9,29,31
511	.48	15° 10° 10°	15° 10° 25°		18,14,12	38,40,36
554	.54	10° 15° 15°	15° 20° 15°		12,34,28	12,17,21
503	.57	15° 25° 15°	15° 15° 20°		43,20,11	23,25,18
038	.42	20° 20° 10°	15° 15° 10°		12,14,24	20,17,11
368	.44	20° 20° 25°	20° 10° 15°		15,17,9	36,14,20
348	.55	20° 15° 20°	40° 15° 10°		9,18,40	29,13,39
245	.58	30° 30° 10°	15° 10° 10°		27,26,9	42,17,27
244	.60	25° 30° 25°	40° 15° 30°		21,26,25	29,33,54
243	.61	15° 60° 30°	35° 10° 20°		29,9,43	10,34,32
242	.62	45° 15° 20°	10° 15° 20°		12,23,17	10,52,32
241	.63	40° 30° 25°	50° 30° 25°		25,8,14	36,43,12
Control. Orientation in the absence of light or any temperature gradient						
		105° 97° 82° 104°				
					34,11,35,57	

any temperature gradient in the agar, at least that was demonstrable with the thermocouples. Corning color filters 2" square (unpolished stock thickness) having different absorption spectra (see fig. 2) were taped to the slot in front of the crystallizing dishes.

In these experiments it was possible to measure the angle of orientation of each pseudoplasmodium. To do this a line was drawn through the center of the dish in the direction of the light source. If the pseudoplasmodia were parallel to this line, the angle was considered to be  $0^\circ$ , indicating perfect orientation. If the pseudoplasmodia were at right angles to this line then they were labeled as having an orientation of  $90^\circ$ . There are 4 quadrants of  $90^\circ$  each and if the angles of the pseudoplasmodia within each quadrant are measured with a protractor, then by disregarding their sign the mean orientation may be calculated. Random orientation would give a mean angle of  $90^\circ$ .

The results of these experiments are given in table 2. It can be seen that no matter what part of the spectrum (between ca.  $0.38$  and  $0.70 \mu$ ) is isolated its radiation is capable of orienting the pseudoplasmodia.<sup>6</sup>

An experiment was also run on intensity discrimination. First attempts were made to determine the maximum distance in which the pseudoplasmodia would still orient towards a  $7\frac{1}{2}$  watt tungsten filament lamp. At 13 feet orientation was still perfect and because of space limitations in the dark room another line of attack was found more practical. Two  $7\frac{1}{2}$  watt lamps were placed 8 feet apart and square dishes (constructed with flat glass bound at the corners with construction tape) were used to hold the 2% agar and the organisms. These were placed at varying distances between the two bulbs and the angles of orientation of the pseudoplasmodia to the bulbs were directly determined. In figure 3 one can see that random orientation ( $90^\circ$ ) is approached directly on the mid-line be-

<sup>6</sup> A complete experiment was also performed with an argon arc obtaining the same result, but the data are not included here since the relative intensities of the radiations at different wave lengths were not known.



tween the two bulbs, and as one approaches either bulb, the pseudoplasmodia progressively approach perfect orientation ( $0^\circ$ ). Again there is no heat gradient in the agar at least that could be demonstrated with the thermocouples.

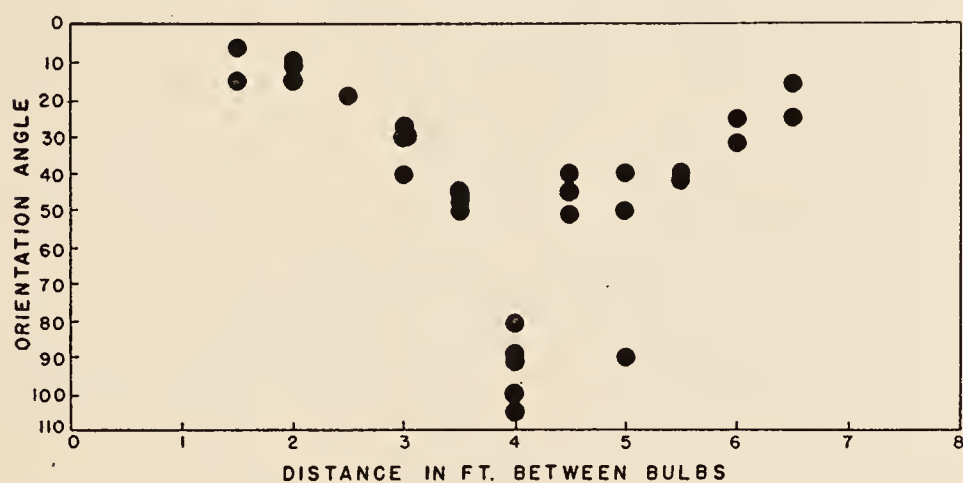


Fig. 3 Graph showing the orientation of pseudoplasmodia placed between two  $7\frac{1}{2}$  watt tungsten filament lamps. The lamps were placed 8 feet apart and on the abscissa of the graph they are located at 0 and 8 feet. The angle of orientation (see text) is given on the ordinate, where  $0^\circ$  is perfect orientation towards the nearest lamp and  $90^\circ$  is random orientation. Note that the random orientation is only approached when the pseudoplasmodia are placed directly between the two lamps.

#### DISCUSSION

It is possible by experiment to obtain independently orientation of the pseudoplasmodium in *Dictyostelium* to heat and to light for there is orientation to a heat gradient in the dark and orientation to light in the absence of a demonstrable heat gradient. And now we must ask ourselves do these separate effects involve two mechanisms, or are they one and the same? This all depends on whether or not the light effect is a photochemical reaction, or whether it is merely a matter of the slight warming of one side of the pseudoplasmodium by absorption, which in effect would amount to a heat gradient. There is unfortunately no way of settling this point, but the fact that it has been impossible to show any spectral limits

to the light effect (since a great range of wave lengths are effective) suggests that no specific photochemical reaction is involved, but rather that there is differential heating on the two sides of the pseudoplasmodium.

The extreme sensitivity of the pseudoplasmodium to heat also helps this hypothesis in that the amount of heat obtainable from light absorption is by rough calculation commensurate with the heat differences capable of orienting. If we consider the thickness of a relatively small migrating pseudoplasmodium to be approximately 0.1 mm in diameter, then in a gradient of  $0.05^{\circ}\text{C./cm}$  which is known to orient them (fig. 2), they will have a difference in temperature between the two sides of the pseudoplasmodium of  $0.0005^{\circ}\text{C.}^7$  So far as we know there is no match for this in the literature of orientation to heat in other animals and plants. There is good evidence that there is a diffusible agent (acrasin) responsible for the aggregation of myxamoebae (Bonner, '47) but we have not been able to orient the aggregating myxamoebae with heat. Although it seems therefore unlikely that heat (which diffuses) is responsible for aggregation, in both aggregation and migration the cells show great sensitivity to diffusion gradients. Unfortunately, at the moment, it is beyond our grasp to understand the mechanism whereby migrating pseudoplasmodia discriminate between such small differences in temperature.

#### SUMMARY

In the amoeboid slime mold *Dictyostelium discoideum* independent amoebae stream together to form sausage shaped cell masses that crawl about the substratum before differentiating into spore and stalk cells. Previously K. B. Raper showed that this migrating cell mass or pseudoplasmodium

<sup>7</sup> Some experiments were performed to see if any special region of the pseudoplasmodium was sensitive, by placing a small spot of light on different regions. Orientation towards the light only took place when the light spot touched the anterior end of the migrating pseudoplasmodium. But it is wrong to conclude from this that only the anterior end is sensitive. It may be that all other regions are physically constrained and only the tip is capable of moving and exhibiting a response to the stimulus.

oriented to light and heat. In an effort to study these orientations quantitatively it was found that if the temperature gradient is as small as  $0.05^{\circ}\text{C./cm}$ , the pseudoplasmodia will orient toward the warmer end. This means that in small pseudoplasmodia the temperature differences between the two sides is in the neighborhood of  $0.0005^{\circ}\text{C}$ . By using various colored filters attempts were made to find the limits of the action spectrum for the light response, but no limits were demonstrable and the pseudoplasmodia oriented towards the light in all cases. On the basis of this experiment an hypothesis was suggested, namely, that this orientation is not caused by a specific photochemical reaction but merely by differential heating in that the illuminated side would be slightly warmer than the darker side, sufficiently to give orientation.

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## THE PATTERN OF DIFFERENTIATION IN AMOEBOID SLIME MOLDS \*

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In the latter part of the nineteenth century Hans Driesch discovered the remarkable fact that if parts of a sea urchin embryo were isolated, then each separate part would produce a perfect, complete, diminutive individual. He called such a system harmonious and equipotential; harmonious because each part somehow was integrated to become a whole, and equipotential because all the parts of the original embryo proved upon isolation to be capable of giving rise to all the differentiated structures of an individual larva. It is an unfortunate historical fact that Driesch is remembered not so much for his deep insight into the problem of regulatory development, but for his philosophical conclusions. He found this kind of development so astounding and so remote from human experience that for him the only possible explanation was a vitalistic one, involving an entelechy. The very fact that the embryo can be subdivided and each part be a harmonious equipotential system proved in his eyes that no mechanistic interpretation could account for the facts, for what machine, he said, will reorganize to make up for the loss of essential parts? But nowadays our reverence for machines has increased many fold and the mathematicians talk of electronic calculating machines that play good chess and can perform various types of involved thinking—a far cry from the mechanical toys that so impressed Descartes. If Driesch had seen these remarkable achievements of today he might have been restrained from his entelechies, although he still might have asked whether cutting a huge calculator in two will give two perfect dwarf calculators. No doubt the mathematicians have an answer, but it is not my purpose here to discuss regulation in electronic calculators.

Instead, I wish to discuss an organism, *Dictyostelium discoideum*, which more than any other living form exposes the problem of regulation. The basis for this rather sweeping statement lies in the very nature of its life cycle, for regulation takes place in a mass of cells which has arisen by aggregation, and not in a cell mass that arises directly by growth and cell division from an egg. As will be shown the cells of the mass may arise from one or many clones and the only factor which determines their ultimate differentiation is their position within the mass, following the well-known principle established by Driesch.

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<sup>1</sup> The new experimental work presented in this paper was carried out with the help of a grant from the American Cancer Society and was also supported in part by funds of the Eugene Higgins Trust allocated to Princeton University. I also would like to record my indebtedness to Mrs. Evelyn Frascella for her able assistance in this work.

The spores of *Dictyostelium discoideum* consist of small, smooth capsules each of which contains a single uninucleate amoeba and upon germination the capsule splits and the amoeba emerges. (See Raper, 1935, 1940a; Bonner, 1944, for details of the life cycle.) This amoeba will feed by engulfing bacteria and as it increases in size it will divide by binary fission so that in the course of time there will be many separate independent amoebae in this vegetative stage. When the food supply is depleted, and even more important when a critical concentration of amoebae is reached, they will begin to aggregate, begin to stream together to central collection points, and apparently the amoebae are guided by a gradient of a chemical substance called acrasin to which they respond chemotactically (Bonner, 1947). The cell aggregate assumes a sausage shape usually 1 or 2 mm. long, and proceeds to migrate over the substratum for variable periods of time depending on the external conditions.<sup>2</sup> At the end of migration the sausage rights itself and pushes up into the air (culmination stage) to form a fruiting body consisting of two cell types: the apical ball of capsulated spore cells and the thin tapering stalk made up of large vacuolate cells enclosed in a smooth cylinder of cellulose.<sup>3</sup> It will now be helpful to trace the steps involved in this final differentiation in more detail.

By the use of some ingenious coloration experiments, Raper (1940b) was able to determine the normal fate of various parts of the migrating cell mass. He found that the anterior portion gives rise to the stalk and the posterior region gives rise to the spore mass. Later it was found possible (Bonner, 1944), by the use of stained paraffin sections, to see in advanced migrating cell masses differences in the appearance of presumptive spore and presumptive stalk cells; the former were small and stained densely with haematoxylin, and the latter were larger and paler after staining. The most remarkable aspect of these sections was that the division line between these early spore and stalk cells was extremely sharp. (See Bonner, 1944, fig. 1.) Following the subsequent stages in the paraffin sections one could clearly see that these prestalk cells first became true vacuolated stalk cells in the tip and that this tip was pushed like a wedge downward through the center of the presumptive spore mass. I have seen this process clearly in a recent experiment in which the anterior cells were stained with the pigment of *Serratia marcescens* (using the technique of Raper, 1940b), and the pushing down of the wedge may be followed (fig. 1). The remaining presumptive stalk cells push upward to the tip of the stalk and as they arrive in this apical position they become trapped and soon enlarge and become vacuolate. So by a combination of the progressive piling of cells on the top of the stalk and their vacuolization the stalk rises into the air. Attached to the apical end of the stalk remains the spore mass, the majority of the

<sup>2</sup> Studies on the factors affecting the duration of migration are now being carried out in collaboration with Mrs. M. K. Slifkin. Methods have been devised to keep migration going for a period as long as 20 days in one instance.

<sup>3</sup> There is also a basal disc peculiar to *D. discoideum* containing vacuolate cells which surround the base of the stalk.

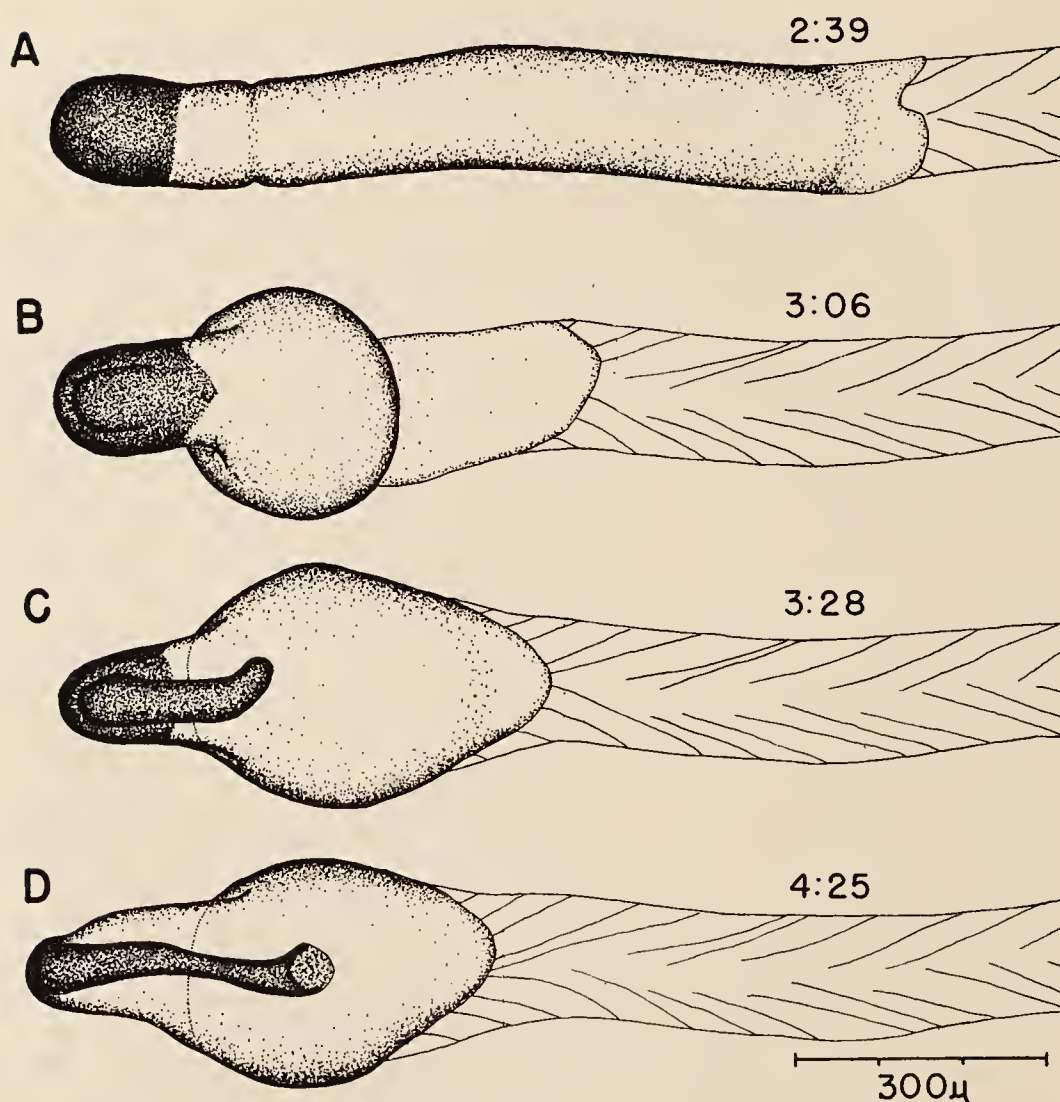


FIGURE 1. Camera lucida drawings (surface views) showing how the stalk is first formed at the tip and is pushed downward through the pre-spore cells to the substratum. The dark tip was obtained by grafting the tip of a colored migrating cell mass onto a decapitated colorless one.

pre-spore cells having differentiated into true spores rather early in the rise upward.<sup>4</sup>

During this past year a new method of observing the division line between presumptive stalk and presumptive spore cells during migration has been found. The advantage of this particular method is that early differentiation may be observed in the living condition. We simply suspend in water large quantities of vegetative amoebae and stain them with a vital dye; neutral red, Nile blue sulphate, and Bismarck brown being the most useful ones.<sup>5</sup> Since all the amoebae are stained in the stage before they have aggregated

<sup>4</sup>The culmination process has recently been examined by Raper and Fennell (in press).

<sup>5</sup>The same results were obtained when the amoebae were stained with the red pigment of *Serratia marcescens* using the technique of Raper (1940b).



the suspension picks up the stain uniformly. The cells are then washed free of the dye solution by centrifugation and placed on plain agar. After some hours they aggregate and form migrating masses that are of an even color which is to be expected since the separate cells took up the stain equally. But with the beginning of differentiation, which may appear immediately at the end of aggregation or a few hours later, the anterior region of presumptive stalk remains dark, while the posterior presumptive spore region in a matter of 10 to 15 minutes blanches considerably (fig. 2).<sup>6</sup> The division line between these two regions is invariably clear and sharp. It is, of course, an assumption that this line actually separates pre-spore and pre-stalk cells, but the

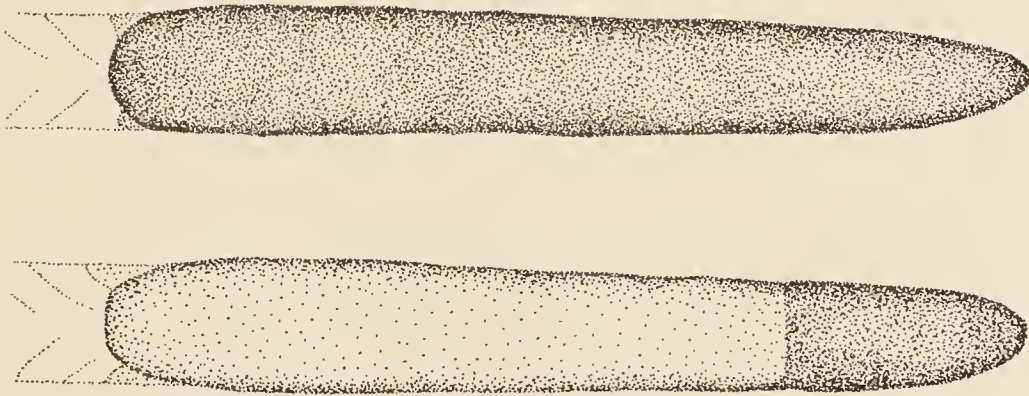


FIGURE 2. Drawing showing how a vitally stained migrating cell mass of uniform coloration (above) will alter to one possessing a dark tip and a light posterior portion (below).

position of the line correlates so perfectly with the separation point of these two tissues that it is hard to imagine it being other than the reflection of the beginning of differentiation.<sup>7</sup> This method may tell one little of the mechanism of differentiation and the separation of the two cell types, but it does show in a striking way how sharply and how suddenly this division may appear. The question now arises as to what extent this division line maintains equal proportions of stalk and spore material in different fruiting bodies.

It must always be kept in mind that starting with aggregation there is no further size increase and that differentiation takes place within a mass of fixed size. The evidence for this is that all food can be eliminated by centrifugation of the amoebae and the cycle is normally completed without the intake of energy. Size then in *Dictyostelium* is dependent entirely on the mass of cells that comes to one collection point during aggregation, and therefore, it is not surprising that the variation in size among individual aggregates is normally great.

<sup>6</sup> It must be assumed that these color changes do not involve a loss in the dye or a redistribution, but a change in chemical combination in different parts.

<sup>7</sup> If the migration continues for some time the staining becomes irregular and blotchy and is no longer consistent in its pattern except that the very tip will always remain intensely stained.

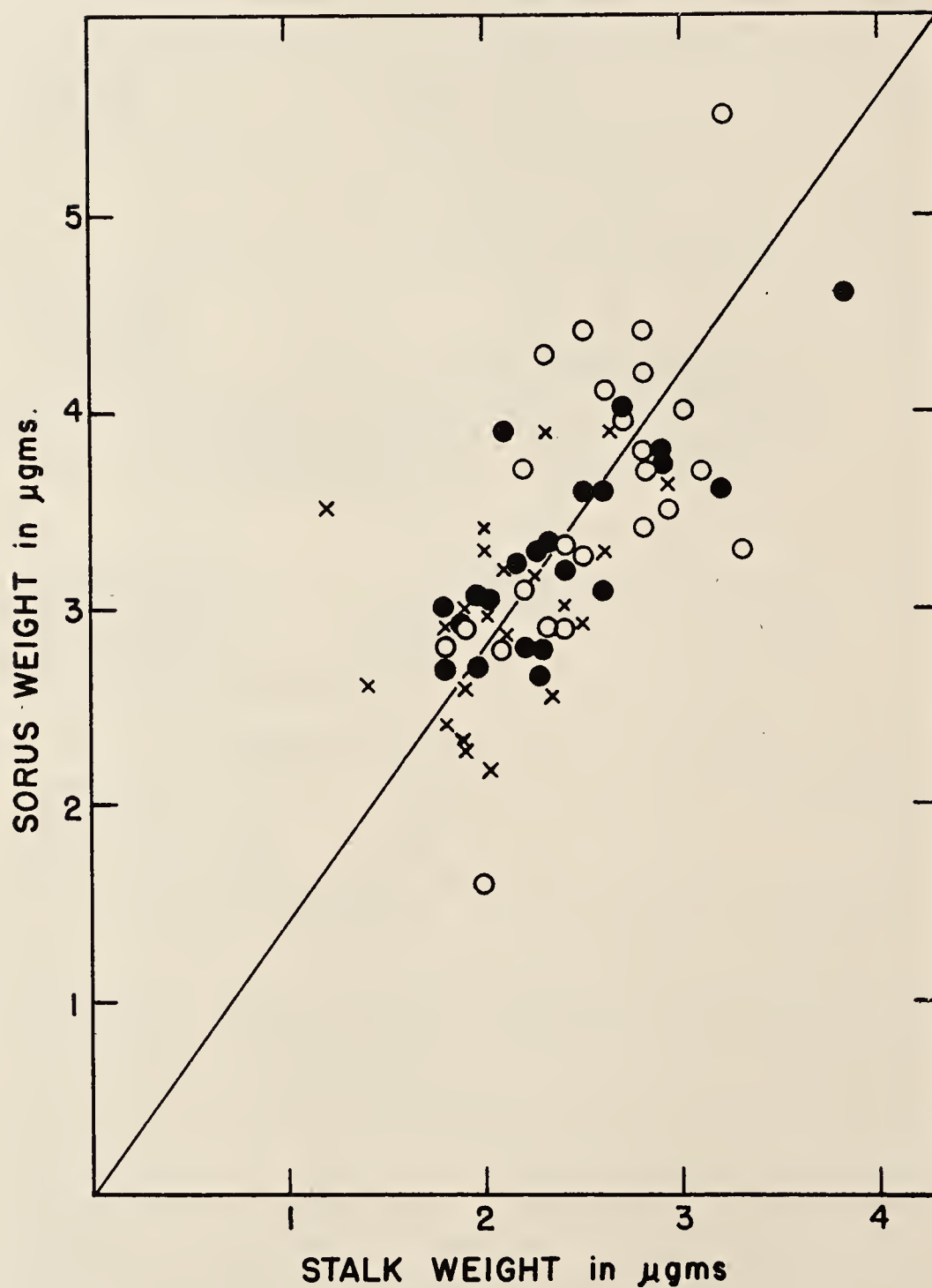


FIGURE 3. A graph in which the dry weight of stalks are plotted against the dry weight of spore masses or sori. Each point indicates a mean value for 30 fruiting bodies from one culture dish. Hollow circles are for those that have fruited at 17°C in the dark; solid dots at 27°C in the dark; crosses at 17°C in the light of a 15 watt "fluorescent" lamp.

If, for a number of different fruiting bodies, the volume of the stalk is plotted against the volume of the spore mass or sorus, then it is seen that with fruiting bodies of different sizes the volume proportions remain approximately constant (Bonner and Slifkin, 1949). The proportions by volume of individuals fruiting under different environmental conditions showed consistent differences, although for any one condition the proportions also remained constant with fruiting bodies of different sizes. In some more recent work I have repeated these experiments using dry weights as a measure. For this a delicate quartz helix balance was used with an average of 30 fruiting bodies to obtain one weight measurement (fig. 3).<sup>8</sup> The curious thing here is that the dry weight proportions remained constant even in different environments, indicating that the volume changes observed previously are most likely caused by great differences in water content arising in the different environments. But the pertinent point to our argument here is that there is a great constancy in the location of the division line between stalk and spore cells.

Let us now examine more closely the equipotential aspect of *Dictyostelium* and see if the different parts may produce wholes. As a first approach individual cells may be isolated and from these a clone may be grown and its subsequent development followed. A number of workers showed that separate clones invariably gave normal fruiting,<sup>9</sup> but now Sussman (1951) has done a careful and extensive study on just this point. He finds that single spores and single amoebae from any stage of development will give rise to a clone that shows normal development in every respect. Therefore, provided a cell may pass through a period of growth and cell division it is capable of giving rise to stalk and spore cells.

The matter of equipotentiality may also be tested during later stages without any reversion to the vegetative stage. The migrating cell mass is perfectly adapted for isolation experiments and Raper (1940b) has amply explored the many possibilities. He has shown that longitudinal fission is a relatively frequent and normal event and both halves produce normal fruiting bodies. Also occasionally fusions take place naturally, giving one large normal fruiting body, but this occurs only when the anterior ends collide so that the axes of the two cell masses are perfectly aligned.<sup>10</sup> If aggregation streams are cut he again found each portion to give a normal fruiting body. Lastly, if a migrating body is cut transversely all the posterior sections give fruiting bodies of normal appearance and we have corroborated this point by making careful volume measurements of the proportions of various pieces of migrating cell masses (Bonner and Slifkin, 1949). The anterior portion will also give a fruiting body but often it has a disproportionately large stalk, a matter which we will discuss again shortly. From this it is

<sup>8</sup> The sensitivity of the balance was such that 1 mm deflection indicated a weight of 9.12  $\mu$ gms. I am indebted to Dr. James H. Gregg (who first used this method on *Dictyostelium*) for kindly allowing me to benefit from his experience.

<sup>9</sup> Raper (1951). Also D. D. Perkins (unpublished) has had similar results and I have done this successfully a number of times myself.

<sup>10</sup> The matter of polarity in fusion is extremely important (Raper, 1940b; Bonner, 1950).



evident that it is possible to make presumptive spores turn into stalk cells and vice versa, again clearly demonstrating equipotentiality. Recently, I have followed these changes using the vital dye technique described above, and if one makes the transection just at the division line between the blanched posterior section and the dark anterior section, then soon afterward the posterior end will again acquire a dark tip and the anterior section will begin to blanch at its posterior end.

The matter of the proportions upon fruiting of the tip fraction is a bothersome question. I am attempting to find what factors affect the proportions, but at the moment there are no satisfactory answers and considerable confusion and contradiction in the results. It is true, as Raper (1940b) first showed, that often the stalk is large in proportion to the spore mass, indicating that there is sometimes in the very tip some degree of "determination" and that the presumptive stalk cells have to a certain extent a fixed fate. If this is so then it might be that our statement concerning equipotentiality is not so all-inclusive and that the tip, at least in some instances, appears mosaic in character. But the tip has many unusual properties that are lacking in the whole remainder of the cell mass and it may be profitable to examine the tip in more detail.

By the tip I mean approximately the anterior  $\frac{1}{20}$  of the cell mass. It is not the whole area of the presumptive stalk cells, but just the anterior-most area. In the first place it was shown previously (Bonner, 1949) that acrasin, the substance responsible for aggregation, was also present in the later stages and that the high point of its production is always at the tip. Furthermore, there is no correlation, no corresponding change, between the acrasin production along the axis of the cell mass and the division line between spore and stalk cells. Also this tip region is in a sense an inductor region, as Raper (1940b) showed, for if a number of tips are grafted laterally on one large migrating cell mass all the tips share the cell mass approximately equally, so that if three new tips are grafted in, four small cell masses will arise. The tip then is an "organization center" although it must be remembered there is no evidence for or against the notion that acrasin is an inductor substance. These properties, then, and possibly also the sensitive photo- and thermo-tropism of the cell mass<sup>11</sup> are properties of the tip alone and do not in any way help us to understand the factors which divide the spore and stalk cells into such stable ratios.

These special tip qualities, however, do again raise the question of whether perhaps there is at the tip a special cell or group of cells (a "queen bee") that is mosaic, that is predetermined at aggregation. After all, the first cells to come into the aggregation center will be those at the tip of the migrating cell mass, and they could have special properties from the very beginning. This hypothesis breaks down for culmination in that during the process of stalk formation the apical cells (which produce acrasin) are constantly changing, constantly becoming trapped, and vacuolate with new cells piling on top of them. But could not this mosaic hypothesis apply for aggregation and migration?

<sup>11</sup> See Bonner, Clarke, Neely and Slifkin (1950).

A few months ago I did some experiments which give some additional information on the nature of the tip. The anterior ends of migrating cell masses stained with a vital dye were grafted into the posterior end of a colorless migrating mass. If the colorless mass is decapitated, as Raper (1940b) had done, then it reorganizes, balls up and attaches itself to the hind end of the anterior colored fragment to form a normal migrating cell mass with a colored anterior end. However, if the colorless host was left intact there was a totally new result: the colored anterior piece slowly moved up the colorless migrating mass so that in a few hours it had achieved a forward position and the whole anterior end was colored (fig. 4). This occurred as the whole mass migrated so that the colored anterior portion travelled at a faster rate than the migrating colorless portion. Furthermore, if a colored posterior piece was grafted into the anterior end it moved backward into the posterior position as migration proceeded. Likewise, anterior portions grafted into anterior positions and posterior portions into posterior positions remained in place.<sup>12</sup> This suggested that the difference between anterior and posterior was one of rate of migration and that the anterior cells were simply there by virtue of being fast. The idea, then, is that migration in the cell mass may represent some sort of Maxwellian velocity distribution and that speed determines position.

The notion that all cells might not move at the same rate is somewhat contrary to the classic idea of migration, for Raper (1940b, 1941) states that if a migrating cell mass half white and half colored is made by grafting, the sharp division line remains throughout migration (6-12 hours) without any conspicuous mixing of the cells. We have repeated these experiments using Nile blue sulphate, neutral red, and using the method involving the red bacterium *Serratia marcescens* and found that, while a distinct division line does remain often for two or so hours of migration, many individual colored cells can be seen to move rather rapidly either forward or backward. In fact, if the cell mass migrates for one or two days it will be evenly colored throughout, but it is quite likely that after such prolonged migration simple physical diffusion also operates.

The evidence then favors the hypothesis that the individual cells each have their own velocity and that the process of migration itself is one in which these different cells are constantly under a process of selection. Only the fastest will be stalk cells and the slowest will be spore cells. This still does not help explain the proportionality that exists between the amount of stalk or spore material, unless one had some critical velocity necessary for stalk-cell formation. But even then it would be hard to see how regulation could take place in a piece isolated by transverse section that gives normal proportions. If there really is a selection here of some

<sup>12</sup>These experiments usually gave rise to abnormal fruiting bodies. A number of controls were run in which no graft was made but a cell mass was lacerated with a needle in a fashion similar to the way it must be in order for the graft to take and similar abnormalities appeared. Those that migrated for long periods after the operation were always normal.

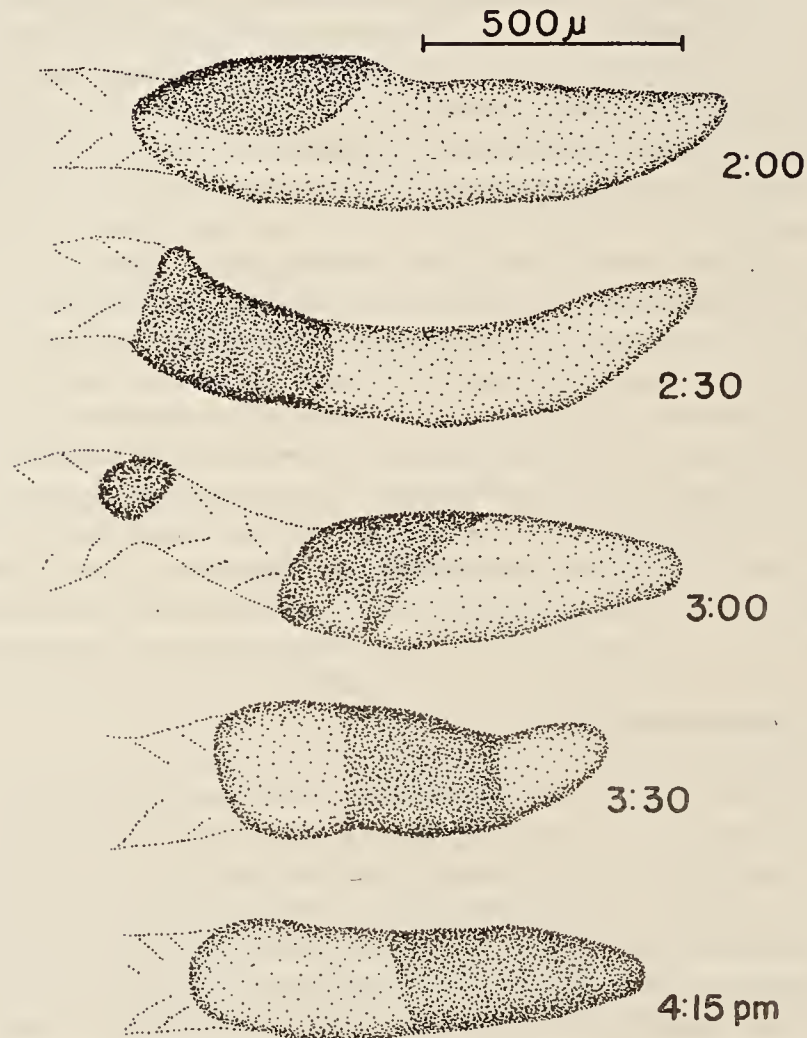


FIGURE 4. Camera lucida drawings showing the rapid forward movement of a colored anterior portion which has been grafted into the posterior region of an intact migrating cell mass. Note that in the middle drawing a piece of the graft was lost.

mosaic character (reflected in the speed of the amoebae) then it should be possible to test this hypothesis by artificial selection.

This was done by taking a migrating cell mass and cutting off a small section of the anterior end. We allowed this anterior portion to fruit and its spores were used to start an  $F_1$  generation. When a migrating sausage was produced in this  $F_1$  then it was also isolated and its anterior end separated just as before. This was repeated until the  $F_{10}$  generation and each of the generations produced normal fruiting bodies which strongly suggests that no selection has taken place. Both posterior and middle fractions of migrating masses were also selected for in the same way for five generations and again no change in the fruiting bodies before and after this repeated selection could be detected. So it would seem, from the present evidence, that even though there be differences in rates among amoebae in the mass,



this does not reflect any mosaic basis of differentiation and the parts of the cell mass are equipotential.

If we now review the salient facts that have been presented we see that in this aggregate of cells there is a division line between the two cell types and that this division line is proportional irrespective of the number of cells in the aggregate. This proportionality cannot be at the moment understood in terms of the tip for the peculiar activity of that apical region, which includes induction and the production of acrasin, does not coincide geographically with the division line between spore and stalk cells. Each fragment of the cell mass appears equipotential and will produce (if it lacks it) a new division line, which may involve stalk cells changing to spore cells and vice versa, and a new tip region. The most stable entity that cannot be altered is the polarity, for the antero-posterior axis remains fixed and cannot be altered experimentally (Bonner, 1950).

When one looks over these facts as I have presented them here it is alarming to realize that even though we know some details of the development of *Dictyostelium*, and a great many details concerning the regulatory development of other animals, we understand no better than did Driesch the explanation of a harmonious equipotential system, and yet a half century of embryology has elapsed. It may be that we no longer find entelechies a temptation, but our substitutes are poor indeed. All we can say is that there are physical analogies that might help us and this thought keeps us confident that ultimately physics and chemistry will not fail us in an explanation. And the only thing we may pretend that our glance at *Dictyostelium* has done is to expose, to dissect out the problem so that possibly it may be seen more distinctly—so that possibly it may keep us from forgetting that one of the most important problems of morphogenesis is still unsolved and still with us.

#### SUMMARY

The following original experimental material is presented in this paper.

1. If the separate amoebae of *Dictyostelium discoideum* are stained with a vital dye the migrating cell mass will first be of a uniform color but later the anterior portion will remain dark and the posterior portion will blanch. The sharp division line corresponds to the position of the division line between presumptive stalk and spore cells. If such a cell mass is cut transversely at the division line, the posterior portion will again become dark at the tip, and the anterior portion will blanch at its posterior end.

2. If the dry weight of the stalk material is plotted against the dry weight of the spore mass a strict proportionality exists even under different environmental conditions.

3. By marking groups of cells with vital dyes and by grafting them in different regions of migrating cell masses it was shown that anterior cells move at a relatively faster rate than posterior cells; if marked anterior cells were placed in the posterior portion, in the course of a few hours they moved up to the anterior end as the whole mass migrated. Likewise, marked pos-

terior cells placed in the anterior end would fall back to a posterior position.

4. It could be seen directly, using vital dyes, that individual cells or groups of cells moved faster or slower than their neighbors, and that movement during migration did not involve a uniform speed of all cells.

5. If anterior fractions of migrating cell masses were each independently selected for in repeated generations, by allowing them to fruit, sowing their spores, and isolating an anterior fraction in the next generation, after a number of such generations the fruiting bodies produced were similar and normal. This was also true for middle and posterior fractions indicating that there is no mosaic segregation taking place during migration.

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## MITOTIC ACTIVITY IN RELATION TO DIFFERENTIATION IN THE SLIME MOLD *Dictyostelium discoideum*<sup>1</sup>

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### TWO FIGURES

In the Acrasiales or amoeboid slime molds, there is, in their development, first a period of active feeding, growth and cell division (the vegetative stage) followed by the aggregation of the cells and subsequent morphogenesis of the collected cell mass or pseudoplasmodium. It is well known that these morphogenetic stages can occur in the total absence of food and for this and other reasons it has always been assumed that no cell division or mitosis occurs after the vegetative stage.

For instance in 1902, Olive says, “. . . no nuclear changes have been observed in the individuals, nor have any indications of increase in numbers by division of the myxamoebae been seen, from the first period of aggregation up to the maturation of the fructification.” In 1926 Harper says, “The ontogenetic processes of cell growth and multiplication are thus sharply separated from those of morphogenesis . . .” In 1941 Raper says, “Beyond this point (aggregation) there is neither an increase in size nor in the number of individuals and all further development consists entirely of the integration and subsequent differentiation of myxamoebae already present.” In 1947 Bonner says, “At the end of this so-called vegetative stage, the myxamoebae cease to feed or multiply,

<sup>1</sup> This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.



thus having a natural separation in their own life histories between growth processes, and purely formative morphogenetic processes.”

It so happens, as will be shown in this study, that cell division does occur after aggregation and furthermore it bears a definite relation to the problem of differentiation.

#### METHODS

The amoebae were grown on standard buffered peptone-dextrose medium (Bonner, '47) and fed with *Escherichia coli*. After two days' growth the amoebae were separated from the bacteria by centrifugation (see Bonner, '47, for the procedure). The washed amoebae were then stained with vital Nile blue sulphate (Bonner, '52) and placed on non-nutrient, 2% agar, at 20°C. (in the dark).

After some preliminary attempts using sectioned material and various nuclear stains, it was decided that the most reliable information on nuclear activity was obtained with aceto-orcein smears.<sup>2</sup> When a particular pseudoplasmodium had reached a desirable stage of development, first a camera lucida drawing was made of it in the living condition and then it was placed for one minute in a fixative of 7 parts glacial acetic acid and three parts methyl alcohol. This was followed by a solution of 3% orcein in 70% acetic acid for 5 minutes, and then smeared in fresh aceto-orcein. Before smearing the pseudoplasmodium was cut into two or three sections (depending on the stage of development) so that the anterior presumptive stalk cells could be separated from the middle and posterior presumptive spores.

On the 6th to 8th day after smearing the preparation was examined with a 95 $\times$ , oil immersion lens and 8 $\times$  oculars. A square grid (Howard eyepiece micrometer) was placed in one of the oculars which covered the actual area on the smear of .012 mm<sup>2</sup>. The number of mitoses (no discrimination was

<sup>2</sup> We are greatly indebted to Professor K. W. Cooper, who not only has been enthusiastically urging us for some years to make this study, but has guided and helped us on both techniques and cytological interpretations.

made between the various stages of mitosis) per  $.012 \text{ mm}^2$  was counted for as many different fields as possible on the preparation, usually somewhere between 20 to 60 fields for each of the segments of the pseudoplasmodium. The number of mitoses per  $.012 \text{ mm}^2$  was then averaged.

It is fully appreciated that as a quantitative method this is very poor indeed since the pressure of the smearing will seriously affect the total number of cells per  $.012 \text{ mm}^2$ . In some cell counts that were made the average was found to be close to 100 cells per  $.012 \text{ mm}^2$ , but the range extended from



Fig. 1 Camera lucida drawings of metaphase plates found in migrating pseudoplasmodia of *D. discoideum*.

approximately 60 to 140. This great variability has been offset in a minor way by counting a large number of samples, but it cannot be obliterated and it means that the results must be interpreted with caution. Only major differences are valid, and for each difference a variance analysis was made.

#### RESULTS

In the mitotic figures observed in the migrating pseudoplasmodium of *Dictyostelium discoideum* the chromosomes possessed 7 large arms (fig. 1). Upon observing a great number of metaphase plates it appeared that these probably represent 4 chromosomes; one with the spindle attachment at or near one end, two with spindle attachments close to the middle, and a 4th with the attachment region about one-third of the way from one end. This would suggest a haploid condition, and the same has been observed in late vegetative cells before aggregation.

Unfortunately it has been impossible, thus far, to obtain an estimate of the mitotic rate during the vegetative stage. The reason for this is that the bacteria ingested by the amoebae also stain with orcein, making it hard to find even occasional mitotic figures.

After centrifugation of the vegetative amoebae, mitotic activity appears to virtually stop and remains so for as long as 12 hours, essentially through all the early part of the aggregation stage (table 1 and fig. 2).

As aggregation proceeds and a visible aggregate begins to rise into the air, the time after centrifugation no longer

TABLE 1  
*Mitotic rates of amoebae after centrifugation.*

HOURS AFTER CENTRIFUGATION	MEAN MITOSES PER .012 MM <sup>2</sup> OF SMEAR
2	0, .06, .08
4	0, 0, .08
6	.02, .05, .07, .08
8	0, 0, 0
10	0, 0, 0
12	0, 0, 0

becomes a suitable index of the stage of development because of the extreme variability in time to achieve the identical stage. Therefore a morphological index was used, namely the height of the forming cell mass over its width (measured at the base). This fraction will steadily increase as development proceeds, at least until it reaches a value of three, any time after which it may fall over and begin to migrate.

The mitoses first begin in the anterior portion, that portion which rises into the air first, and that portion which is destined to contribute the stalk cells to the final fruiting body. It is followed shortly by considerable activity in the posterior two-thirds or presumptive spore region. That the anterior region does actually precede the posterior region is shown



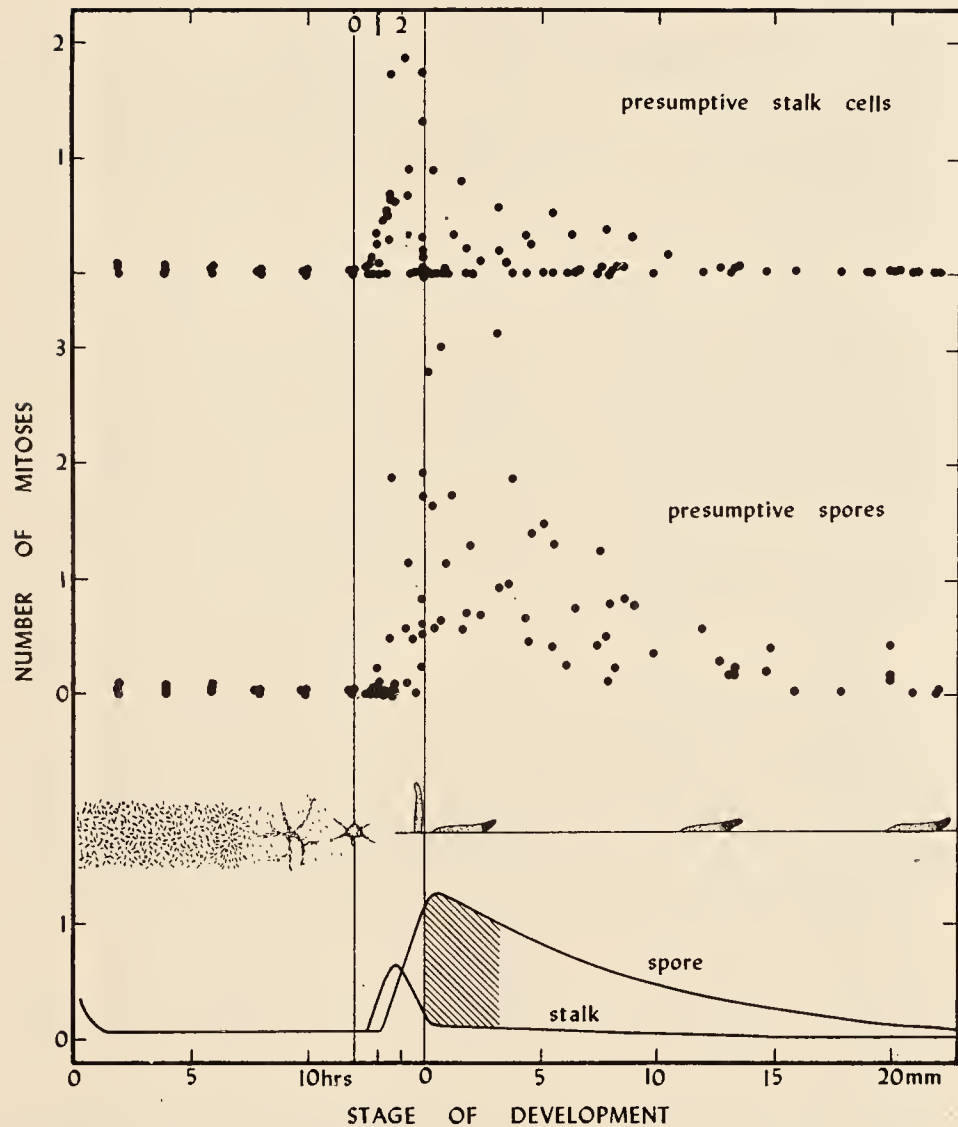


Fig. 2 Graph showing the approximate mitotic rates of different parts of pseudoplasmodia at various stages of development. The ordinate is the number of mitoses per  $.012 \text{ mm}^2$  of the smear preparation. The abscissa is divided into three scales. The first is hours after centrifugation, the second the ratio height over width of the aggregating pseudoplasmodium, and the third is distance of migration in millimeters. A diagram is included to serve as a rough guide of the various stages of development. The top graph shows the actual data for the anterior presumptive stalk cell region, the second graph shows the data for the posterior presumptive spore region. The lower curves are a diagrammatic summary of the two upper graphs and because of the inaccuracy of the method of measuring the mitotic rates this should not be used except as a visual aid in observing differences between the top two curves. The shaded region indicates the period where, evenly stained blue pseudoplasmodia may become two-toned in appearance, as indicated in the drawing above.

TABLE 2  
*Mitotic rates at the end of aggregation*

HEIGHT/WIDTH OF THE CENTRAL MASS	MEAN MITOSES PER .012 MM <sup>2</sup> OF SMEAR	
	Anterior $\frac{1}{3}$	Posterior $\frac{2}{3}$
.62	.06	0
.76	.07	0
.80	.16	0
.80	0	0
.90	0	0
1.04	.34	.06
1.04	.26	.23
1.10	0	.09
1.10	.09	.08
1.33	.45	0
1.50	0	0
1.52	.53	0
1.55	.52	0
1.60	.30	.48
1.60	.67	0
1.60	.64	0
1.66	1.72	1.85
1.86	.62	.19
2.31	1.86	.57
2.40	.67	.11
2.40	.89	1.12
2.56	0	.47
2.71	0	0
3.00	0	.86
3.00	1.73	1.81
> 3	0	.52
> 3	.15	1.90
> 3	.24	.23
> 3	0	.82
> 3	1.31	.59

in table 2 and figure 2 (between the two vertical lines).<sup>3</sup> But the activity in the presumptive stalk region is short lived and already at the beginning of migration it has fallen abruptly, while the posterior region is steadily rising.

<sup>3</sup> A statistical analysis was made of this point and it was found that if the anterior values (between 0 and 1.7 height/width) are compared with the posterior values, the anterior ones are significantly higher ( $p < 5$  by the "t" test). We are indebted to R. F. Link of the Department of Mathematics at Princeton University for assistance on these statistical problems.

The stage of development during migration was best measured by the distance of migration. The anterior third soon loses its very slight activity, while the posterior two-thirds rises to a peak early in its migration, and the mitoses do not stop until 20 to 25 mm of migration have taken place <sup>4</sup> (table 3 and fig. 2).

As had been shown previously (Bonner, '52) if the vegetative amoebae are stained with vital Nile blue sulphate, the migrating pseudoplasmodia are first evenly stained, but later there is a difference in the intensity of the stain: the anterior end corresponding with the presumptive stalk region is dark blue and is sharply separated from the light blue posterior two-thirds. This transition from a uniform blue to a two-toned condition was variable in its appearance, the range being zero and slightly over 3 mm of migration <sup>5</sup> (see the shaded area in fig. 2).

No mitoses were ever observed during early stages of culmination, when the first signs of stalk formation appeared. This was not only true of those pseudoplasmodia that had migrated long distances, but also of those that culminated immediately following aggregation, during the period where ordinarily they would exhibit the highest mitotic activity.

#### DISCUSSION

We may then, without hesitation, say that mitotic cell divisions are frequent and common following aggregation in

<sup>4</sup> Since the rate at 20°C. very roughly approximates 1 mm per hour (Bonner, Koontz and Paton, in press) this would mean that in the spore region mitoses continue for approximately 20-25 hours.

A statistical analysis was made and it was found that during migration the anterior one-third had a significantly higher mitotic rate than the average of the middle one-third and the posterior one-third. ( $p < 1$  by the "t" test.)

Also an analysis was made to see if the middle fraction differed significantly from the posterior fraction for the mean of the middle fraction is higher. The difference is possibly significant ( $p$  between 5 and 10 by the "t" test) which could be explained by the fact that in the very posterior end there are cells which do not become spores and therefore might lower the rate.

<sup>5</sup> A small series was run without centrifugation and without Nile blue sulphate to be sure that neither of these two factors were affecting mitosis, and these controls showed no differences from the experimentals in their mitotic activity.



TABLE 3  
*Mitotic rates during migration*

MM OF MIGRATION	MEAN MITOSES PER .012 MM <sup>2</sup> OF SMEAR			
	Anterior $\frac{1}{3}$	Middle $\frac{1}{3}$	Posterior $\frac{1}{3}$	Middle + Posterior
0	.30	.46	.56	.51
0	0	2.62	.77	1.69
0.3	0	2.50	2.95	2.73
0.5	.88	2.13	1.07	1.60
0.6	0	.59	.53	.56
0.8	0	.50	.77	.64
0.9	0	3.59	2.68	3.14
1.0	0	1.36	1.00	1.18
1.3	.34	.77	1.86	1.32
1.7	.80	1.09	0	.55
1.9	0	1.10	.24	.67
2.1	0	1.45	1.12	1.29
2.5	.11	.19	1.17	.68
3.2	.56	3.80	2.30	3.05
3.3	.19	.93	.88	.91
3.6	.09	1.06	.86	.96
3.9	0	1.90	1.70	1.80
4.4	.33	.88	.40	.64
4.5	0	.41	.44	.43
4.7	.25			1.39
5.2	0	1.32	1.59	1.46
5.6	0	.33	.44	.39
5.6	.52	1.40	1.00	1.20
6.2	0	.31	.17	.24
6.5	0	.75	.74	.75
7.5	0	.52	.28	.40
7.7	.06	1.76	.72	1.24
7.9	.36	.13	.72	.43
8.0	0	0	.20	.10
8.0	0	.85	.73	.79
8.3	.05	.06	.35	.21
8.7	.06	.88	.76	.82
9.0	.30	.70	.77	.74
9.9	0	.37	.31	.34
12.0	0	.63	.45	.54
12.8	.04	.17	.37	.27
13.2	0	.15	.16	.16
13.2	0	.17	.15	.16
13.4	.05	.24	.18	.21
14.7	0	.18	.17	.18
16	0	0	0	0
18	0	0	0	0
19	0	.13	.23	.18
19	0	.17	.32	.25
20	0	.13	.17	.15
20	0	.13	.06	.10
20	0	.38	.40	.39
21	0	0	0	0
22	0	0	0	0
22	0	0	0	0
29	0	0	0	0
30	0	0	0	0
35	0	0	0	0
35	0	0	0	0
37	0	0	0	0
38	0	0	0	0
42	0	0	0	0
50	0	0	0	0
60	0	0	0	0

the slime mold *D. discoideum* (with the possible exception of cases in which culmination comes immediately after aggregation).<sup>6</sup> As a matter of fact, in some preliminary observations we have found this to be true of all the Acrasiales tested thus far: *D. mucoroides*, *D. giganteum*, *D. purpureum*; *Polysphondylium violaceum*, *P. pallidum*, even though these forms do not possess a migration stage and begin stalk formation immediately.

Of further interest is the fact that in *D. discoideum* the mitotic activity of the anterior presumptive stalk cells differs from that of the posterior presumptive spore cells. The former start first and decline very rapidly while the latter continue for a considerable period of time. This means that a detectable difference between the two presumptive areas occurs as early as the latter part of the aggregation stage, earlier than had hitherto been suspected. The difference revealed by the staining with a vital dye appears somewhat later, and occurs when the mitoses in the anterior region have practically stopped while those of the posterior region are at their peak.

It is well known (Raper, '40) that if a migrating pseudoplasmodium is cut transversely, regulation will take place and each piece will produce a fruiting body. To see if differentiation and regulation were strictly dependent on mitotic activity, migrating pseudoplasmodia that had migrated more than 30 mm, and hence would have ceased all mitoses, were cut into segments. Then at varying times after the operation they were smeared with orcein, but in no case was there evidence of any recurrence of nuclear activity in any of the segments. Therefore, we might assume that mitotic activity only reflects differences in parts resulting from early changes in the differentiation process and the changes do not require mitoses.

<sup>6</sup> Even in these cases it is quite possible mitoses appear in late aggregation before the culmination begins.

## SUMMARY

Contrary to previous reports, good evidence is presented that in the amoeboid slime mold *Dictyostelium discoideum* (as well as in other members of the Acrasiales) there is considerable mitotic activity following the aggregation of the independent amoebae. The mitoses of *D. discoideum* are accompanied by cell division and involve 4 chromosomes made up of 7 arms. The mitotic rates of the presumptive stalk regions differ in time with those of the presumptive spore cells, indicating that they reflect early signs of differentiation. In fact this is the earliest detectable difference between the two presumptive regions. Evidence is presented to show, however, that differentiation is not dependent on mitoses.

## ADDENDUM

Since the completion of this manuscript we have learned of the work of Dr. C. M. Wilson on the cytology of *Dictyostelium discoideum* (Proc. Nat. Acad., in press) and here we shall briefly discuss the similarities and the differences of our findings. In the first place, he believes that each of the 7 arms is a separate chromosome and they are not grouped into 4 chromosomes. But a more important difference is his view that fusion takes place at aggregation and that it is followed immediately by reduction division. If this is so, what we have called mitosis might conceivably be meiosis. We looked carefully for evidence of meiosis during the following aggregation and found none. Perhaps this is because of a difference in technique for we centrifuged our amoebae free of bacteria, while Wilson took them directly from the incubation dish. But whether the nuclear events are mitotic or meiotic does not affect the interpretations made here in this paper concerning differentiation. On the matter of differentiation, Wilson made a significant discovery which we missed, and that is that just prior to the final differentiation into spores there is a rapid wave of mitotic divisions of the presumptive spore cells.



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## SIZE IN RELATION TO THE RATE OF MIGRATION IN THE SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*<sup>1</sup>

JOHN TYLER BONNER, PAUL G. KOONTZ JR., AND DAVID PATON

(WITH 2 FIGURES)

It is characteristic of the Acrasiales or amoeboid slime molds that a group of uninucleate amoebae aggregate, but in only one species described thus far, *Dictyostelium discoideum* Raper, does this cell-aggregate or pseudoplasmodium migrate for a period of time before the final fruiting or culmination. The migrating pseudoplasmodium has a sausage or cartridge shape and moves slowly over the substratum secreting a slime sheath that is deposited as a track posteriorly. It has been studied by a number of investigators<sup>2</sup> and found to have numerous interesting structural and physiological properties, although many aspects of its behavior remain unexplained, including the mechanism of its motion. In order to collect more information concerning this interesting stage of development, a study has been made of the rates of movement and it was found that similar to the rates of culmination (3) the larger the pseudoplasmodium, the greater the speed of migration. As will be shown, this lends evidence to the hypothesis that the propelling force for movement is internal and depends on the total mass of cells rather than a superficial cell layer.

### METHODS

The slime mold was grown in a two membered culture with *Escherichia coli* on the same nutrient medium used in previous studies (2). Prior to migration, when the cells had just assembled

<sup>1</sup> This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.

<sup>2</sup> See Raper (6) for references to the work of Raper, Gregg and Bonner. Also there is some more recent work of Slifkin and Bonner (7).

into pseudoplasmodia, certain of these were transferred with a steel spatula to the surface of a non-nutrient, 2% agar medium. As has been shown by Slifkin and Bonner (7) this medium favors prolonged migration, thereby facilitating prolonged observations on the same pseudoplasmodium. During both the growth and the subsequent migration stages the petri dishes were kept at  $20.5 \pm 0.5^\circ \text{C}$ . (in the dark) and during the migration period the dishes were kept within a closed moist chamber to prevent desiccation.

As soon as migration started a camera lucida drawing was made of the pseudoplasmodium, and this was repeated at approximately 12- or 24-hour intervals. The distance travelled was traced (by following the slime track) with india ink on the bottom of the petri dish. This was later measured by placing the petri dish bottom in a lantern slide projector and tracing the projected track on the screen with a map mileage indicator and making the necessary unit conversions. The volume of the pseudoplasmodia was estimated by the segment method given in Bonner and Slifkin (4). Since

TABLE I  
SHOWING THE RELATION BETWEEN THE VOLUME OF MIGRATING  
PSEUDOPLASMODIA AND THEIR RATE

(The volumes are a calculated mean of the volume before and after a given period of migration for which the rate has been determined.)

Pseudo-plasmodium number		Volume in mm. <sup>3</sup>	Rate in mm./hr.	Pseudo-plasmodium number		Volume in mm. <sup>3</sup>	Rate in mm./hr.
1	1	.0438	2.00	5	1	.0168	.92
	2	.0400	1.75		2	.0153	.59
	3	.0350	1.25		3	.0129	.33
2					4	.0103	.25
	1	.0337	1.25	6	1	.0236	1.63
	2	.0238	1.04		2	.0209	1.14
	3	.0177	.83		3	.0195	.80
	4	.0163	.79		4	.153	.70
3					5	.0126	.68
	1	.0259	1.61	7	1	.0414	1.26
	2	.0224	1.16		2	.0399	1.17
	3	.0155	1.00		3	.0315	.47
	4	.0118	.76		4	.0247	.48
	5	.0101	.72	8	1	.0437	1.33
	6	.0064	.61		2	.0363	1.07
	7	.0048	.53		3	.0255	1.01
4					4	.0217	.63
	1	.0412	1.50				
	2	.0396	1.33				
	3	.0337	.95				
	4	.0245	.91				



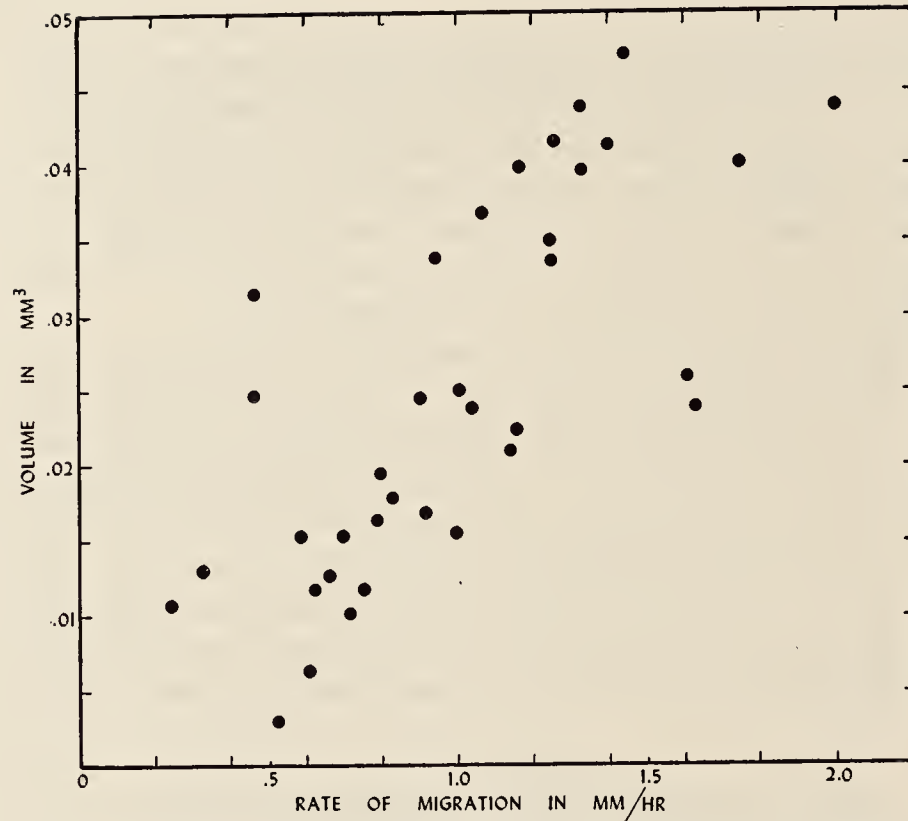


FIG. 1. Graph showing the relation of the volume of migrating pseudoplasmodia (ordinate) to their rate of movement (abscissa).

the rate is given by distance between two observation points (camera lucida drawings) in a given time, the volume equivalent to this rate was estimated by obtaining the mean volume of the two drawings.

#### RESULTS AND DISCUSSION

The results for eight different migrating pseudoplasmodia, are given in TABLE I, and if each average volume is plotted against the rate, it is obvious that the larger the volume, the greater the rate (FIG. 1). This fact is even more clearly seen in an individual pseudoplasmodium, for as migration proceeds the pseudoplasmodium becomes smaller, and its speed is correspondingly slower (see TABLE I and FIG. 2). The reason for the decrease in size during migration is probably two-fold: some of the cells straggle and are lost in the slime track, and also the pseudoplasmodia expend energy without taking in food.

These results parallel previous work on culmination (Bonner and Eldredge, 3), where again the larger the pseudoplasmodium the greater the rate of upward rise into the air. As was pointed out then, the results on culmination, and now we may include the ones given here on migration, fit in with the hypothesis involving the



FIG. 2. *Above.* A series of camera lucida drawings of a single pseudoplasmodium (No. 3 in TABLE 1) showing, from right to left, the gradual decrease in size upon migration. *Below.* A graph of the same pseudoplasmodium in which the ordinate is the volume and the abscissa the rate of movement. (The volume values are each a mean between two of the camera lucida drawings above, corresponding to a particular rate measurement.)

principle of similitude originally proposed by Tyler (8) for a somewhat similar situation in sea urchin embryos.

According to the principle of similitude, in similarly shaped bodies of different size, the volume of surface ratio will increase with size, for volume increases as the cube of the linear dimensions, but the surface as the square. Since large migrating pseudoplasmodia move faster, the locus of the movement force is likely to be dependent on the volume or mass rather than the surface. On the other hand the resistance to movement or friction could be a surface phenomenon. This hypothesis assumes that the power of movement per unit mass of cells is constant for different size pseudoplasmodia, and Gregg (5) has shown that this assumption is supported by the fact that the oxygen consumption per unit nitrogen of tissue is constant irrespective of size.

There has been much speculation concerning the nature of this migration movement and relatively few facts. It is known that the internal amoebae are actively pseudopodial and that the individual amoebae may, to some degree, travel at different rates (Bonner, 1). We may now postulate from the evidence presented here, that all the amoebae are actively involved in the forward progression and somehow gain traction from both the slime sheath they secrete and from one another below the surface of the cell mask.<sup>3</sup> The idea that the forward progression is achieved solely by the outside, superficial layer of cells is not supported by the present evidence.

#### SUMMARY

In the amoeboid slime mold *Dictyostelium discoideum*, separate amoebae aggregate to form sausage-shaped migrating pseudoplasmodia. The rates of movement of these pseudoplasmodia were found to be proportional to their size. This was true even of indi-

<sup>3</sup> There is no objection to the idea that an internal amoeba might obtain traction from his neighbors provided there is an anchor (the slime sheath) somewhere in the system. An amoeba moves by keeping its sides stiff, in a gelled condition, while the internal fluid protoplasm spouts forward at the anterior tip. If the lateral stiff portions of each amoeba stick to one another they will automatically have their traction. Also it is conceivable that each amoeba gives off slime which has some rigidity for traction and is ultimately deposited inside the slime track.



vidual pseudoplasmodia, for the longer one migrated the smaller it became and its rate decreased correspondingly. An hypothesis was presented, based on the principle of similitude, to the effect that migration movement was dependent on the total mass of amoebae, and not upon a superficial layer of specialized locomotory amoebae.

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## VARIATIONS IN CELL SIZE DURING THE DEVELOPMENT OF THE SLIME MOLD, *DICTYOSTELIUM DISCOIDEUM*<sup>1</sup>

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In many organisms, especially multicellular ones, it is relatively difficult to obtain an estimate of individual cell sizes, but in the amoeboid slime mold *Dictyostelium discoideum* it is not only possible to do this with reasonable accuracy for one stage, but for most of the stages of development. In the life cycle of this slime mold (Raper, 1935, 1951; Bonner, 1944) the amoebae are first separate during their actively feeding stage, and later after a short period of fasting they aggregate to form sausage-shaped cell masses which migrate for variable periods of time. During this migration stage the anterior cells begin their differentiation into stalk cells and the posterior cells begin their differentiation into spores (Bonner, 1952) and then finally the migrating mass shoots up into the air on a delicate stalk made up of the anterior cells which now have become vacuolated and encased in a hard cellulose sheath, and at the tip of this stalk there is a globular spore mass made up of cells encased in elliptical spore capsules. The range of individual cell sizes of each of these stages (with the exception of the mature stalk cells which could not be measured accurately because of their irregular shape) was determined and it was found that changes in the stage of development as well as early signs of differentiation were all reflected in characteristic changes of individual cell sizes.

### MATERIALS AND METHODS

The method of culture and the culture medium were the same as those used previously and the aggregating and migrating amoebae were prepared by centrifuging the vegetative amoebae free of bacteria and placing them on plain agar (Bonner, 1947). The cells at each stage were removed and placed in a drop of standard solution (NaCl, 0.60 gm.; KCl, 0.75 gm.; CaCl<sub>2</sub>, 0.30 gm.; distilled H<sub>2</sub>O, 1000 ml.) on a microscope slide. A No. 1 coverslip (22 × 22 mm.) was placed over the drop, but supported on two bits of coverslip so as to prevent the cells from being crushed. The diameters of the rounded, spherical cells were then measured with a 95 X, oil immersion objective and a Zeiss filar micrometer. In the case of the spores, which are elliptical in shape, both the long and the short axes were measured, and the spore diameter is expressed as the mean of these two values for each spore measured.

A number of tests were run to determine if the diameter of an individual cell remained constant when immersed in standard solution. It was found that during a period from 5 to 90 minutes after immersion the cell diameter did remain fairly constant, the small fluctuations being apparently caused by the activity of the contractile vacuole.

<sup>1</sup> This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.

The accuracy is considered to be most reasonable for the vegetative amoebae and the spores. However, during the aggregation and migration stages a fair per cent of the cells either did not round up after being subjected to the salt solution, or they remained part of a mass of cells and were impossible to measure. Therefore, during these two stages, there might conceivably be a sizable error due to having selected only those cells that were spherical. In defense of this it can only be said that there was no apparent or striking size difference and that it is believed that if an error has been introduced by this procedure it is likely to be relatively small.

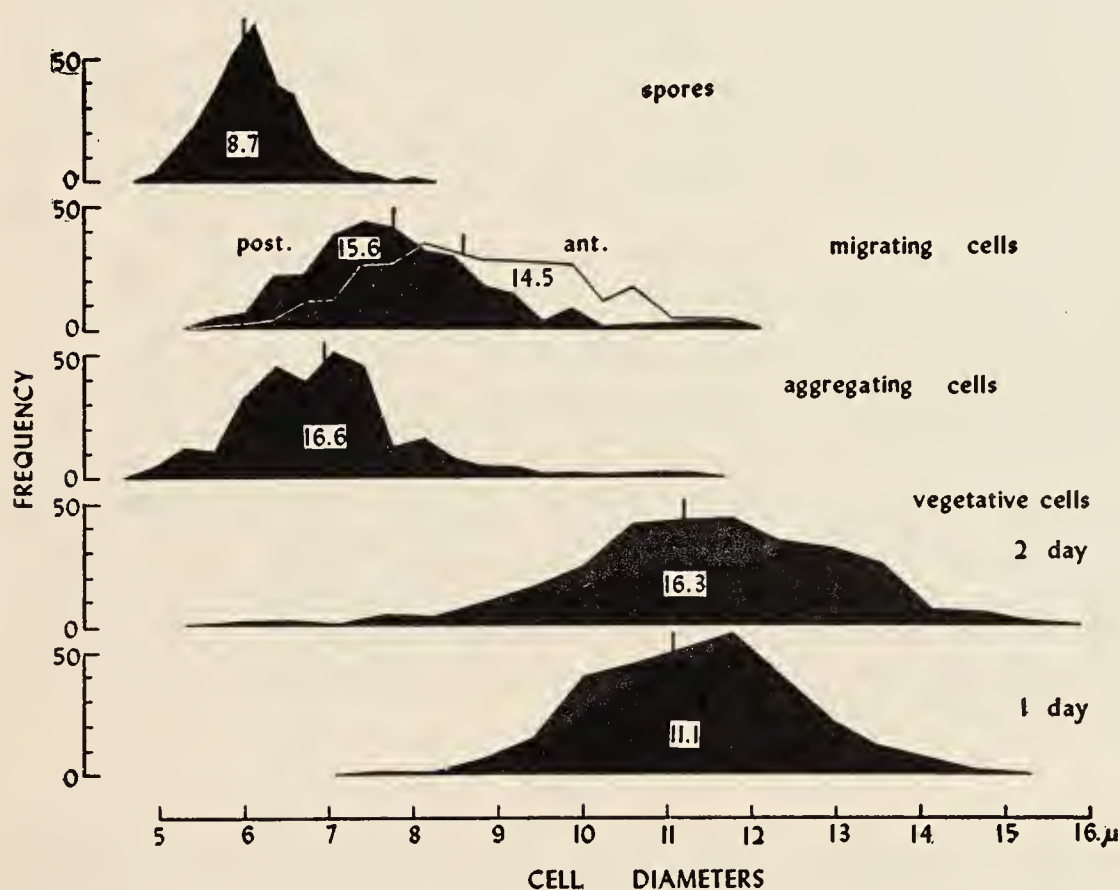


FIGURE 1. A graph in which the frequency is plotted against the cell diameter for various stages of development. Each curve consists of 300 observations. The vertical lines indicate the mean values. The numbers placed within the curves are the coefficients of variation in per cent.

### RESULTS

The diameters of 300 cells were measured for the following stages of development: (1) vegetative amoebae approximately one day old (between 22.5 and 23.5 hrs.), (2) vegetative amoebae approximately two days old (between 41.5 and 46 hrs.), (3) aggregating amoebae, (4) and (5) the anterior and posterior cells at the beginning of the migration stage (3 to 4 mm. of migration) and (6) the mature spores. For each stage, this count of 300 was made on three separate



samples of 100, except in the case of the migrating cells which were measured in four samples (anterior) and five samples (posterior).

An analysis was made of the measurements at each of these stages to determine which of the three quantities, diameter, surface area or volume, would give a more normal distribution when plotted against frequency. It was found, by plotting the cumulative relative frequency against these three quantities on probability paper, that for all of the stages of development measured, the diameters gave the curve with the least skewness.<sup>2</sup> In fact the two vegetative amoebae curves as well as the anterior migrating cells and the spores very closely approximated the normal distribution curves, while the aggregating cells and the posterior migrating cells showed a certain skewness in the higher end of their range.

If the frequencies are plotted against diameters for all the different stages and are shown on one graph, as has been done in Figure 1, it becomes obvious that the mean size (indicated by the vertical lines) goes through considerable fluctuations during the course of development. It remains constant throughout the vegetative period but makes a dramatic drop during the aggregation stage. During the migration period, the cells increase in size, but most interesting here is the fact that the anterior presumptive stalk cells are highly significantly larger than the posterior presumptive spore cells. A spot check was made on 50 anterior and 50 posterior cells of an old migrating cell mass (one that had migrated 25 mm.) and it was found to be the same as the young cell masses tested. The spores, on the other hand, are the smallest of all the stages.

In glancing at Figure 1, one can see that the range varies for each stage and an analysis was made to see to what extent this was related to size, for the larger the mean, the larger would be the expected range if the amount of variation about the mean were the same. To compare the different stages the usual procedure of dividing the standard deviation by the mean was employed to obtain the coefficient of variation. These coefficients are indicated on Figure 1 and it can be seen that during the first day of vegetative growth the variability is low but that it rises during the second day and remains high in the migration stage, only to drop to its lowest value in mature spores.

#### DISCUSSION

It is not surprising to find that the mean size does not change between one and two days of vegetative growth, and the cause of the sudden drop during aggregation is perhaps understandable for the vegetative amoebae are actively feeding and engulfing bacteria, while aggregating cells have been fasting for a considerable period of time. (In this case it has been some 17 to 18 hours since the amoebae were washed free of most of the bacteria by centrifugation.) There is no known explanation why the cells increase in size during the migration stage, but certainly it cannot involve feeding but must involve internal osmotic changes. The most important fact is that in the migrating cell mass the anterior presumptive stalk cells are significantly larger than the posterior presumptive spore cells, giving another example of an early detectable difference in differentiation. These migrating cell masses were stained with vital Nile blue sulfate and show characteristic dark anterior ends and light posterior ends (Bonner, 1952).

<sup>2</sup> The authors are greatly indebted to Dr. M. H. Belz, visiting Professor at the Department of Mathematics, Princeton University, for his help in the statistical work in this paper.

The variability during certain stages is remarkably high in *Dictyostelium* when one compares it to the variability of various unicellular organisms listed by Adolph (1931). To fully appreciate the consequences of this, one need only visualize the volumes; the largest two-day old vegetative amoebae, for instance, may be 18 to 19 times greater in volume than the smallest ones.

In comparing the coefficients of variation of the different stages there is an interesting trend, for the variability is low during the spore stage and rises somewhat after one day of vegetative existence, but only reaches a peak of variability after two days.

It is a curious fact that for these cell populations the diameters should give a normal distribution curve rather than the surface areas or volumes. Of course, since the factors which determine size, variability and skewness are virtually unknown, there is no reason to expect any particular type of distribution curve. An intriguing possibility might be, however, that the linear dimension gives a normal distribution because a ratio of the volume to the surface is in some way limiting to cell size and this ratio is, of course, linear. But unfortunately there is at the moment no way to weigh down such a wild speculation with a few facts.

#### SUMMARY

1. The individual cell size was measured for 300 cells at various stages during the development of the slime mold *Dictyostelium discoideum*; during two periods of the vegetative stage when the amoebae are actively feeding, during the aggregation stage when the amoebae are streaming together to form cell masses, during the migration stage when the cell mass wanders over the substratum, and finally the mature encapsulated spores.

2. The mean size was large during the vegetative period, dropped severely in the fasting aggregating amoebae, increased slightly during the migration stage, only to fall to their minimum size as mature spores.

3. The variability in size was large and especially so during the periods before, during and after aggregation.

4. Of special interest relative to the problem of differentiation was the fact that during the migration stage, the anterior presumptive stalk cells were significantly larger than the posterior presumptive spore cells, being another example of an early detectable sign of differentiation.

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## A HISTOCHEMICAL STUDY OF DIFFERENTIATION IN THE CELLULAR SLIME MOLDS <sup>1</sup>

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SEVEN FIGURES

*Dictyostelium discoideum* may be considered to begin its life history with the germination of its spores. From each of these elliptical capsules emerges an amoeba that feeds by phagocytosis and repeatedly divides in two as it grows. Following this vegetative period the amoebae both become sensitive to and begin to emit a chemical substance called acrasin and this substance, by evoking a chemotactic response in the other amoebae, helps to bring the cells together in fairly large aggregates (Bonner, '47; Shaffer, '53a, '53b). An aggregate assumes a sausage shape and exudes a thin transparent slime sheath which is left behind as it crawls over the surface of the agar. It is known that the cells of approximately the anterior third of the sausage will become stalk cells and those of the posterior two-thirds will differentiate into spore cells (Raper, '40; Bonner, '44). After a period of migration the cell mass assembles into a rounded drop which points upward and the presumptive stalk cells form a central cellulose cylinder in which the stalk cells that lie internally become large and vacuolate, while the remaining presumptive stalk cells climb to the stalk tip, thereby extending the stalk into the air. As this occurs the mass of

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presumptive spores is passively raised by the actively forming stalk tip, and fairly early during the course of this upward journey, each presumptive spore undergoes its final differentiation by becoming encapsulated (Bonner, '44; Raper and Fennell, '52).

In some previous studies using various vital dyes, especially Nile blue sulphate, neutral red and Bismark brown, it was shown that if vegetative amoebae were stained, they took the stain uniformly, principally in the form of small granules in vacuoles, and after aggregation the sausage was evenly colored. But later, during migration, the anterior presumptive stalk region remained dark, while the posterior presumptive spore region was clearly of a lighter intensity (Bonner, '52). A number of other differences between the anterior and posterior regions indicating early signs of differentiation are also known: the anterior region of the sausage emits more acrasin than the posterior region (Bonner, '49); the mitotic activity of the two regions differs (Bonner and Frascella, '52); and the volume of the individual cells in the anterior presumptive stalk region is considerably greater than that of the posterior presumptive spore cells (Bonner and Frascella, '53).

Also it is known that in different size aggregates, despite a considerable variability, the ratio of the stalk cells to the spore cells is roughly constant in any size cell mass and this can be shown by measuring the mature stalks and spore masses (Bonner and Slifkin, '49; Bonner, '52) or by measuring the location of the division line in the sausages containing the vital dye Nile blue sulphate.<sup>2</sup>

Since we have, then, evidence for early proportionate development in the migrating sausage, it was thought that any

<sup>2</sup>This statement is based on some unpublished data in which 157 migrating pseudoplasmodia stained with Nile blue sulphate were drawn (camera lucida) and the relative lengths of the anterior and posterior portions were compared. To contrast this with the previous study involving volume measurements of final fruiting bodies, in the migrating ones, in 157 cases, the coefficient of variation (std. deviation divided by mean) was .40 while in the previous study for 109 cases of final fruiting bodies the coefficient of variation was .34.

information concerning the chemical differences between the two cell types might ultimately lead to a clearer understanding of what basically is involved in this differentiation process. As will be shown we have been rewarded to this extent: We have obtained evidence indicating that the posterior presumptive spore cells are essentially passive, showing no special activity or tendency to consume key substances, but instead they conserve all their materials which are concentrated, condensed and finally stored in capsules ready to start the next generation. The anterior presumptive stalk cells on the other hand are spenders and show a progressive depletion of their reserves, as well as high enzymatic activity. Much of the form of the fruiting body is dependent on polysaccharides; these are involved in the slime sheath and the firm cellulose stalk sheath. It was, therefore, especially interesting to follow the changes in polysaccharides during the life cycle and it was even possible to show in what way the stalk itself is deposited by the cells during morphogenesis.

Once some understanding was obtained of the situation in *Dictyostelium discoideum*, we decided to compare it with a few other representative species of the Acrasiales. In this comparative study it became clear that for each species there is a characteristic timing of the various chemical alterations and these differences in the rates of these processes appear to be correlated with, and possibly even responsible for, the differences in the morphology of the various species.

#### MATERIALS AND METHODS

The method of culture and the culture medium were the same as that used previously (Bonner, '47).

To prepare fixed and stained material at the vegetative or aggregation stages, the "under-water technique" was used (see Bonner, '47, p. 6) which consists of allowing the amoebae centrifuged free of bacteria to settle, in this case on glass coverslips (No. 1). The coverslips were then allowed to remain in the standard salt solution until the amoebae had arrived at the desired stage of development, at which time

the coverslips were carefully removed and placed in the fixative.

Migrating and culminating pseudoplasmodia were obtained either directly from standard nutrient culture plates or from two per cent agar plates after being vitally stained with Nile blue sulphate (see Bonner, '52 for procedure).

A variety of histological and histochemical techniques were used in this study. In the case of vegetative and aggregation stages the techniques were applied directly to the cover slips. Pseudoplasmodia were dehydrated and embedded in paraffin after fixation and then serially sectioned at 6 to 10  $\mu$ .

The following methods were used:

*Feulgen for DNA.* Moderately good results were obtained using Carnoy's fixative, cold acetone ( $-5^{\circ}\text{C}.$ ), 10% formalin buffered to pH 7.0, and Helly's fluid. However, the best preparations were obtained following fixation in Zenker's fluid for two to 4 hours and this fixation was employed in all the cases described. The material was hydrolyzed in 1 N HCl for periods varying from three minutes to one hour. A period of 20 minutes was found to give the best results. Further details of the procedure are given by Gomori ('52).

*Basophilia and Ribonuclease digestion for PNA.* Good fixation was obtained with Serra's fluid for 2 to 4 hours while fixation in Carnoy's fluid was found to be inferior. This is consistent with the experience of Brachet ('53) who discusses the problems of fixation of PNA in some detail. Basophilia was exhibited by staining slides and coverslips with a  $\frac{1}{2}\%$  to 2% solution of toluidin blue O for 20 minutes. After staining the material was dehydrated in alcohol, cleared in xylene, and mounted in clarite. Ribonuclease digestion was carried out using a 0.1% solution of crystalline ribonuclease (Nutritional Biochemicals Co.) in redistilled water adjusted to pH 6.0. The material was digested for one hour at  $37^{\circ}\text{C}.$  and then stained with toluidin blue O as described above. Controls were treated for the same period with redistilled water adjusted to pH 6.0 but lacking any ribonuclease.



*Metachromasia.* Metachromasia was demonstrated by the same staining procedure described above for the demonstration of basophilia following fixation in Carnoy's fluid. Fixation with 4% basic lead acetate gave a much more vivid metachromasia but the quality of morphological fixation was too poor to permit detailed observation. Digestion of the materials with 0.1% hyaluronidase (Nutritional Biochemicals Co. and General Biochemicals, Inc.) for one to 18 hours at 37°C. did not alter the metachromasia.

In the observations which are to follow, it is assumed that all material exhibiting a basophilia which is removed by digestion with ribonuclease is pentose nucleic acid, at least in part. For the validity of this assumption see Brachet ('53). It is not possible at this time to attribute with any certainty metachromasia to a specific group of chemical compounds. For a discussion of the significance of metachromasia and the diverse materials which are metachromatic the reader is referred to Gomori ('52) and Pearse ('53).

*Periodic acid-Schiff reaction (PAS) for polysaccharides:* Following the recommendations of Deane et al. ('46), cold Rossman's fluid was used exclusively for the fixation of polysaccharides. Details of the PAS technique are given by Gomori ('52). Since it is not known if these slime molds possess starch or glycogen, the two terms will be used synonymously in this paper. These polysaccharides were removed prior to periodic acid oxidation by salivary digestion for three hours.

*Best's Carmine for Glycogen:* Glycogen (or starch) was demonstrated in material fixed in Rossman's fluid by the Best's carmine procedure as given by Gomori ('52). The best results were obtained when the staining time was increased to 4 hours. All materials which stained with Best's carmine and have been attributed to the presence of glycogen in the observations below were removable by digestion with saliva for three hours at room temperature prior to staining.

*Alkaline phosphatase:* Alkaline phosphatase was demonstrated in material fixed in cold absolute acetone (—5°C.) according to the revised method of Gomori ('52). Sodium

glycerophosphate buffered to pH 9.4 was employed as a substrate. The incubation period was 4 hours at 37°C. Control slides were incubated in a buffered solution lacking substrate or were placed in boiling water for three minutes prior to testing for enzyme activity.

#### RESULTS

*Metachromasia.* During most of the stages of the life cycle of *Dictyostelium discoideum*, the amoebae, upon staining with toluidin blue, show a number of beautiful metachromatic granules. Usually these granules lie scattered in the cytoplasm, but in the spores they form a tight-knit cluster which lies a short distance from the nucleus. The only stage in the life cycle when these red granules are absent is during the vegetative stage. Shortly after germination an amoeba loses its metachromasia and this is not recovered until the aggregation phase. As aggregation approaches, the number of cells showing metachromasia rises rather rapidly until finally at aggregation all the cells show the red.

Attempts were made to correlate this staining reaction with either acrasin emission or sensitivity to acrasin, but it was possible to show that the metachromasia appears before either of these two phenomena. The acrasin emission can be tested by looking for aggregation patterns, and the sensitivity to acrasin was tested by placing migrating pseudoplasmodia (which are known to be giving off acrasin, Bonner, '49) among the amoebae and seeing whether or not the amoebae orient toward the source of acrasin. Immediately after such a test the amoebae were fixed and stained and metachromasia could be shown to have appeared before the sensitivity to acrasin manifests itself. It is, of course, still possible that there is some connection between metachromasia and the acrasin mechanism, but it is clear they do not arise simultaneously.

One of the interesting facts that emerged during this study was that, if the cells which are for the most part metachromatic are centrifuged just prior to aggregation, then

within one to two hours after this rather rugged treatment the cells all lose their red color and revert completely to the vegetative staining condition. We have known for some time that aggregation never starts until 8 to 16 hours after centrifugation no matter what the previous condition of the amoebae, and here, metachromasia also appears to be affected by centrifugation. As aggregation approaches, the metachromasia slowly creeps back; first in a few cells and then in all. Since the bacteria have been largely removed by the centrifugation there is little or no feeding in this case during the period without metachromasia.

*DNA and nuclear size.* DNA was demonstrated in the nuclei at all the different stages of the life cycle by the Feulgen method. Two types of differences in the nuclei were noted: frequently mitotic figures were observed as expected from previous work (Bonner and Frascella, '52), and there was an obvious size difference in the nucleus at different stages of development which also was somewhat expected since it is known that the cell size varies (Bonner and Frascella, '53). Measurements were made of the nuclear size by making camera lucida drawings of 30 nuclei at each stage and then averaging the major and minor diameters of each. In the previous work on cell diameters cited above there were some technical difficulties, namely, it was impossible to be certain that a representative group of cells was selected since only those that were rounded and free could be measured, and also it was impossible to measure the stalk cells since they were highly irregular polygons.

The data on the nuclear size is shown in a series of graphs in figure 1, and although more complete, the graphs correspond well with the data previously published on cell volume (fig. 1 in Bonner and Frascella, '53). The main difference between the nucleus and the cell measurements is that in the nuclei the size decreases continuously going from the vegetative stage, through aggregation to migration, while the cell volumes show a drastic drop during aggregation and a slight increase during migration. It is not known if this difference is due to



an error in the previous cell volume measurements or whether it represents a real fluctuation of the nuclear-cytoplasmic ratio during aggregation.

In the beginning of migration there is no significant difference between the size of the presumptive spore and presumptive stalk cell nuclei, but later the difference is quite

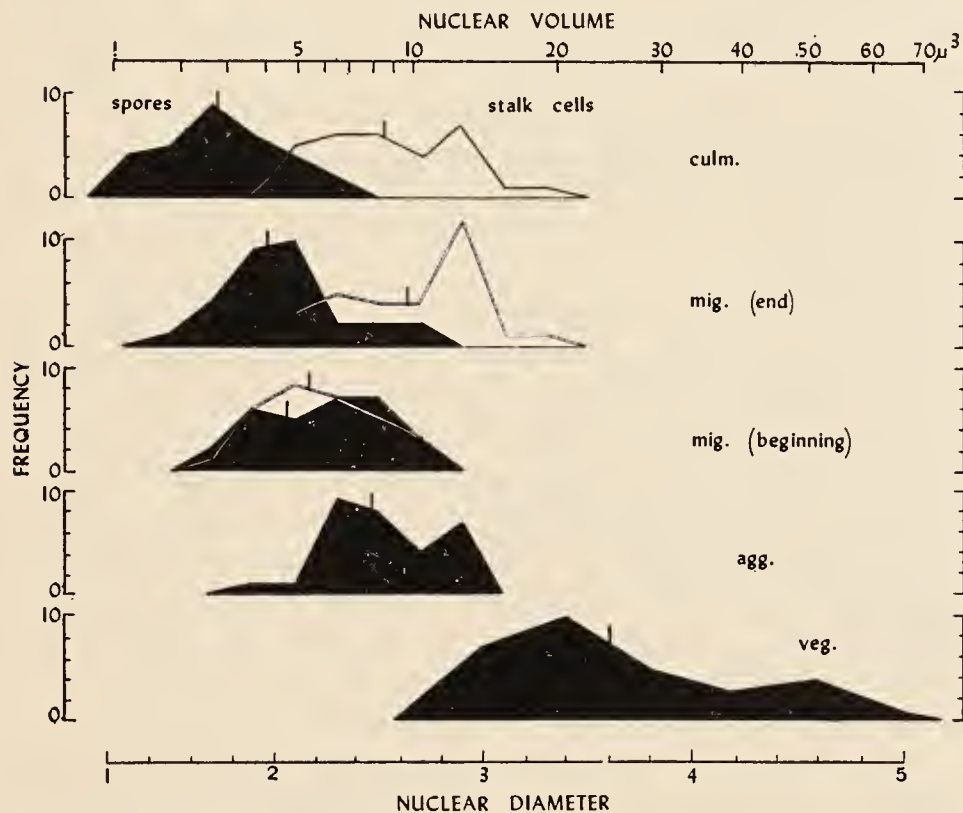


Fig. 1 A graph in which the frequency is plotted against the nuclear diameter for various stages of development. Each curve is based on 30 observations. The vertical lines on each graph indicate the mean values. Veg., agg., mig. and culm. are abbreviations for the vegetative, aggregation, migration and culmination stages, respectively.

evident, and even more exaggerated in the mature stalk and spore cells. There is a continuous trend of decreasing size in the spore nuclei, while the stalk nuclei begin by decreasing in size until the middle of migration when they reverse and increase again.

*PNA.* With the use of toluidin blue and the proper controls with ribonuclease a study was made of the changes in the pentose nucleic acid content of the cells. In the beginning it was thought that there was a difference in the PNA correlated with the early signs of differentiation, that is, that the presumptive spore cells stained darker than the presumptive stalk cells. However, upon repeated trials a number of cases showed no difference and a number showed the reverse situation, that is, the presumptive spores were light and the presumptive stalk cells dark. This extreme variability is hard to understand and one can only conclude at the present moment that there are no clear-cut, consistent histochemically demonstrable differences in the PNA content of these two presumptive cell types.

*Polysaccharides.* In contrast with the PNA studies, it was possible to show clear differences in the polysaccharide content of the different cell types. The most interesting results were obtained after the starch or glycogen had been removed by salivary digestion, and therefore all the information given below is on non-starch polysaccharides. When the starch was selectively stained with Best's carmine it was shown to be evenly distributed throughout the cytoplasm of both the presumptive stalk and spore regions, and, therefore, it was uniformly masking the more important polysaccharides revealed by the periodic acid-Schiff method.

During both the vegetative and the aggregation stages the polysaccharide is found distributed both diffused throughout the cytoplasm as well as concentrated in small granules (fig. 2). The highest concentration of the red staining bodies usually appears localized on one side of the nucleus, but this region bears no relation to the polarity of movement of the cell and might either lie in front or behind the nucleus, a fact especially obvious during aggregation.

At the end of aggregation or at the beginning of migration all the cells appear fairly uniformly stained, at least the two presumptive stages are indistinguishable. In some instances there is a more intense stain along the edge of the cell mass,

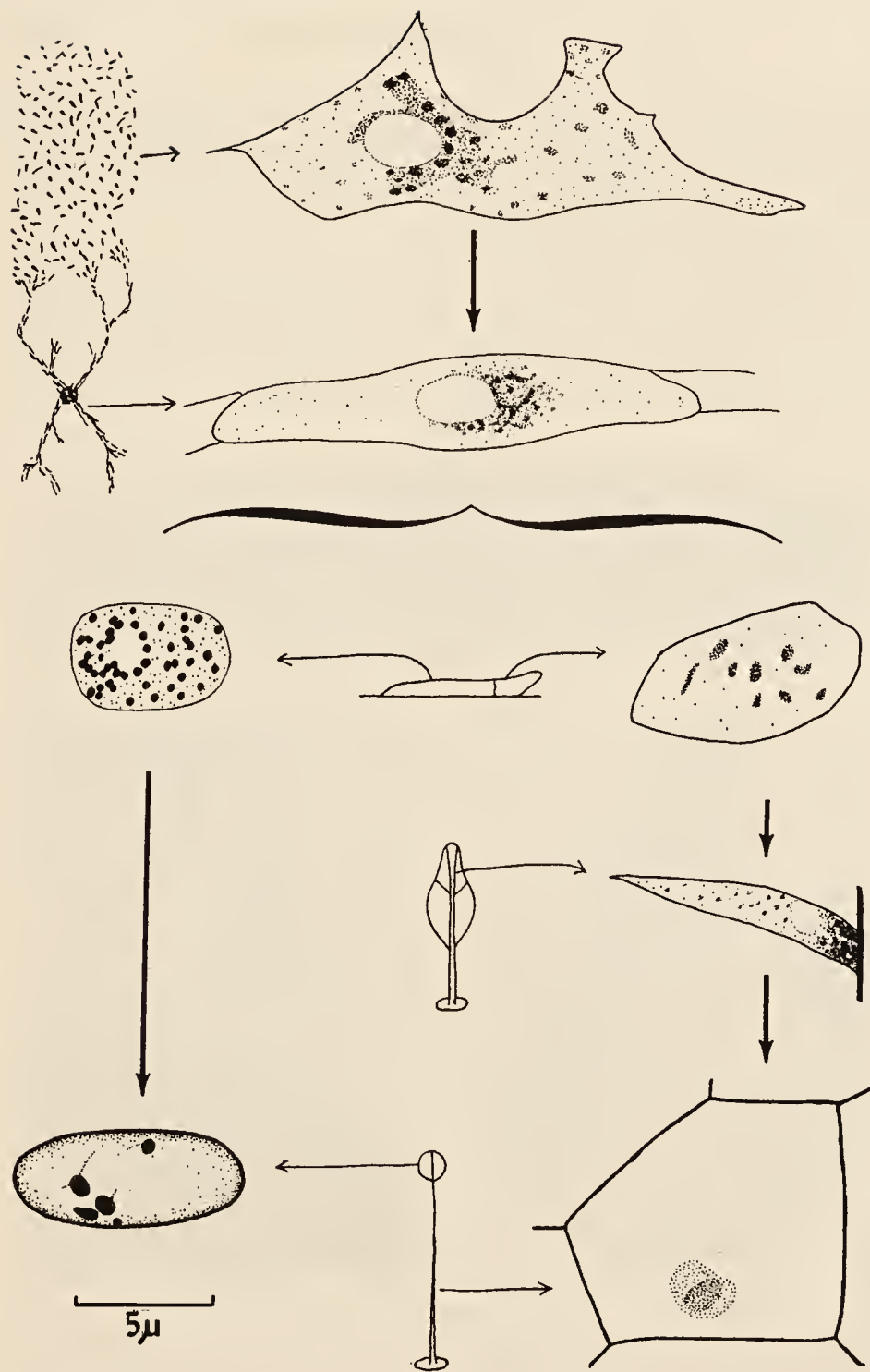


Fig. 2 Camera lucida drawings indicating the distribution of non-starch polysaccharides in the cells at different stages of development of the life cycle of *D. discoideum*. The small diagrams at the upper left and lower center indicate the stage at which the cell was fixed and stained.



especially at the tip region, but there is no assurance that this is not an artifact (fig. 3a). It should be mentioned here that the thin slime sheath, which encompasses the whole cell mass, is definitely PAS positive.

As migration proceeds a sharp difference arises between the presumptive spore and presumptive stalk cells. The spores show a series of small uniform granules that are intensely stained, whereas the stalk cells are much lighter in appearance,

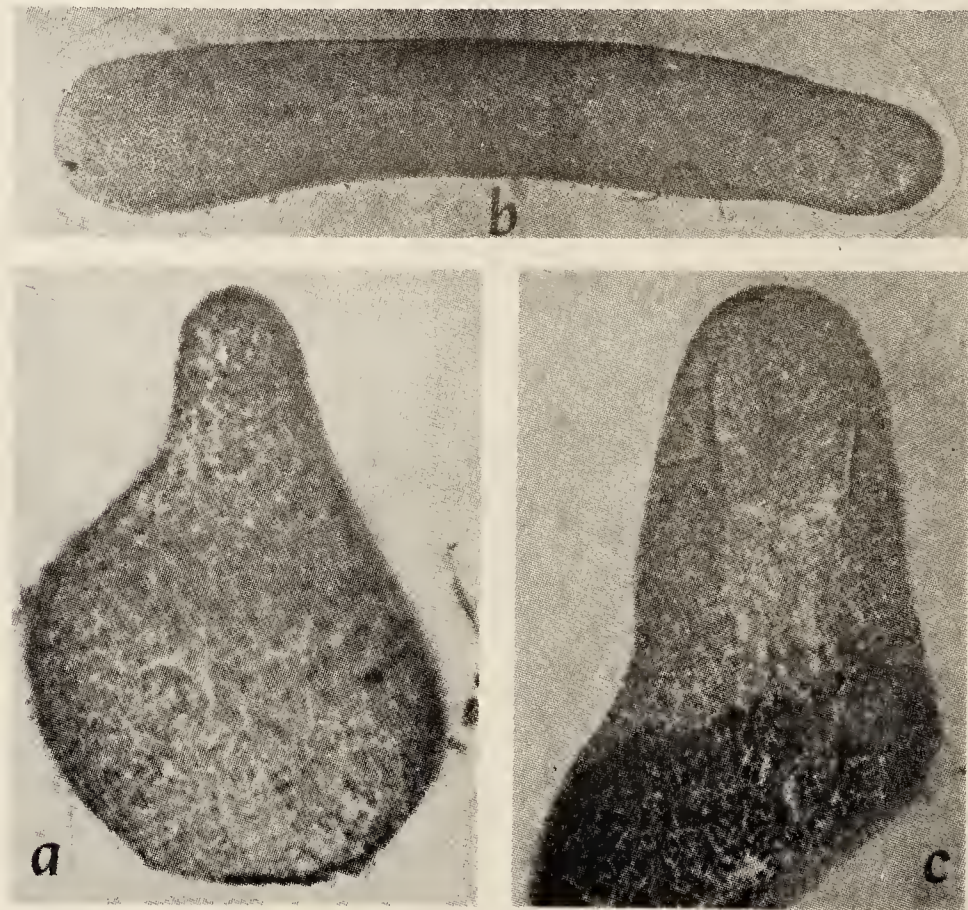


Fig. 3 Photographs of sections prepared by the PAS method showing the distribution of the non-starch polysaccharides. *a*, at the end of aggregation, showing an even distribution of stain; *b*, late migration showing the dark posterior presumptive spore region and the light anterior presumptive stalk region; *c*, early culmination again showing the difference between the presumptive stalk and spore region as well as the zone of deposition of the cellulose sheath which surrounds the final stalk.

having a diffuse color and one or a few large irregular red granules. The line of demarcation (as seen in the low power of the microscope) between the two tissues is sharp and most often runs directly across the sausage-shaped cell mass, although occasionally the stalk cells form a trailing wedge that penetrates into the spore mass. This difference appears to correspond with the previously mentioned differences shown *in vivo* by the use of vital dyes and it also corresponds with the difference revealed by hematoxylin some years ago (Bonner, '44).

A study was made to determine whether this line of demarcation remains stationary during the course of development or whether it moves so that presumptive stalk cells would become presumptive spore cells, or vice versa. By two independent methods it was definitely established that at least until the beginning of culmination the position of the line is fixed. In one experiment 61 migrating sausages stained with Nile blue sulphate were drawn *in vivo* with a camera lucida. On each drawing not only was the line of demarcation indicated but also the length of the slime track to show the distance migrated. Then the length of the presumptive spore region and the length of the presumptive stalk region was measured and it was possible to plot the ratio of these lengths (i.e., spore/stalk) against the distance of migration. There was a fair scatter of the points, as expected from previous work on proportions (Bonner and Slifkin, '49) but there was clearly no trend, that is, the proportions did not become altered with age and increasing duration of migration. In another experiment 6 migrating cell masses and 7 masses just beginning culmination (with a stalk running through the cell mass) were fixed, embedded in paraffin, sectioned, and prepared by the PAS method. These particular cases were selected because each contained a well centered medial section and this section was drawn with a camera lucida and measured with a planimeter. Then the ratio of the presumptive spore area over the presumptive stalk area was determined at the two different stages. The migrating masses showed a ratio



of 1.76 with a standard deviation of  $\pm 0.53$ , while the early culminating masses were  $1.74 \pm 0.54$ . With such small samples it is no doubt pure chance that the fit is so close, but, nevertheless, we may feel confident of the result because the two separate experiments say the same thing, namely, that the division line remains fixed until the beginning of culmination and does not change from its initial position in the cell mass.

As differentiation proceeds in the spore cells, the color of the PAS reaction becomes increasingly intense and the final result is that the spore capsule itself is darkly stained and inside there are either a few or a number of granules, depending on the fixative. These granules in the spores appear similar to the metachromatic granules of the spores, but at no other stage is there any apparent correspondence between PAS positive granules and metachromatic granules.

There is an especially interesting situation in the case of the stalk cells. As the cells move anteriorly to become incorporated into the stalk, they become elongate in a radial direction almost perpendicular to the stalk. When they pass a certain specific region of the stalk, large quantities of polysaccharide accumulate at the inner end of the cell and are liberated at this point (fig. 2, 3c). This, then, is the actual process of the stalk formation, and these cells, which are lining up as a transitory glandular epithelium are actively secreting the stalk. Once the cells have given off the bulk of their polysaccharide they pass upward to the tip and become trapped within the stalk cylinder itself. Then, as they slowly expand and become vacuolate, they show, besides red cell walls, a few large and moderately faint red granules.

It was shown previously (Bonner, '52) that in the case of Nile blue sulphate, if a migrating cell mass was transected at the division line between the dark front and the light hind end, then in the course of time, as the two bits migrated for a period before fruiting, the all-light posterior piece turned dark at its front end and the all-dark anterior piece blanched at its hind end. This seemed to indicate that the dye could be bound in two different ways and that there was a readily



reversible pathway from the light to the dark phase and vice versa.

The very same situation appears to apply to the polysaccharide staining. In 8 migrating pseudoplasmodia stained with Nile blue sulphate as an indicator, a whole section surrounding the division line was removed and discarded and in 4 of the 8 the separate anterior and posterior pieces were fixed after one hour (20°C. in the dark migrating on non-nutrient two per cent agar) while the remaining 4 were fixed after 6 hours' migration. In most of the cases after one hour some evidence of reversal was evident (i.e., the deep red blanching at the tip of the posterior segment and the faintly stained cells turning deep red at the hind end of the anterior piece), while in six hours a fairly complete reversal had taken place in the staining of the appropriate zones, and in some cases normal culmination (with the normal zones) was already in progress (fig. 4). It would appear, then, that the difference in the polysaccharide reaction of the presumptive stalk and spore regions is not caused by a loss of carbohydrate in the anterior portion, but rather is the result of a reversible metabolic change of which one side of the reaction reveals more stainable polysaccharide than the other. The important point here is that whatever are the normal chemical pathways, they are all reversible until the final differentiation of mature spores or stalk cells has taken place.

*Alkaline phosphatase.* The vegetative and aggregating amoebae were found to possess alkaline phosphatase but there was no way of accurately assessing whether the amount present in either of these two stages was significantly different from the other. It was also present in the migrating pseudoplasmodia and what is most interesting is that it appears with different intensity in different parts of the cell mass. In early migration a small area of the extreme tip shows intense phosphatase activity and then in later migration the whole anterior end — that which corresponds to the presumptive stalk — is extremely active compared to the relatively inert posterior portion (fig. 5). During culmination

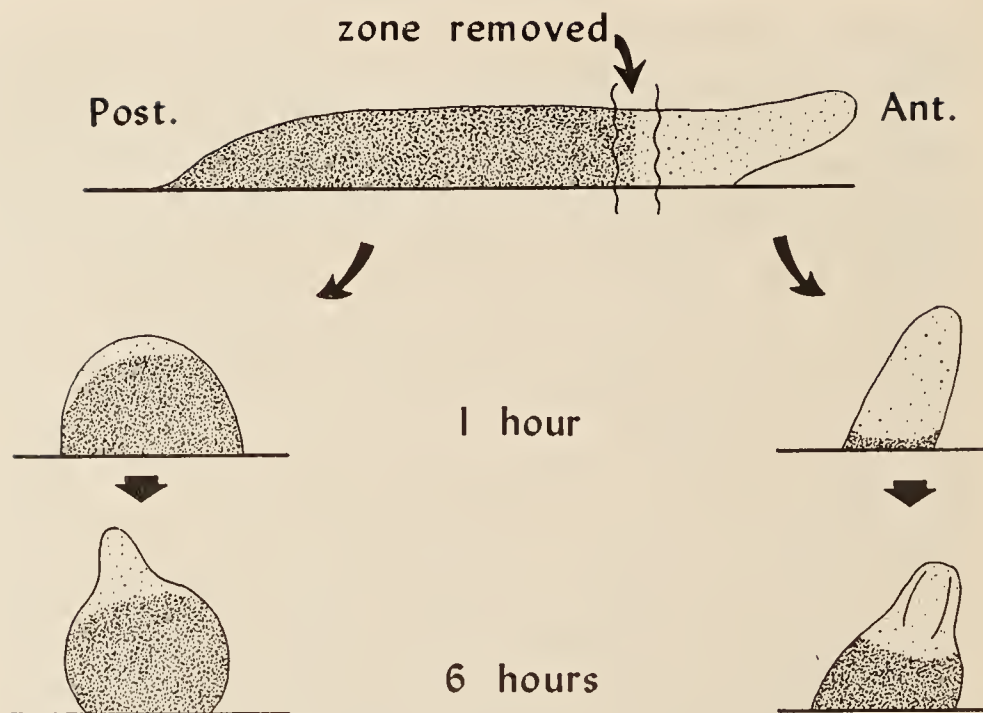


Fig. 4 A diagram illustrating the experiment in which a partially differentiated migrating cell mass is bisected and each portion is examined by the PAS technique after one and 6 hours, respectively. Note that the anterior end of each fragment reversed its PAS staining properties; in one case from the light pre-stalk condition to the dark pre-spore condition and vice versa in the other.

the activity in the presumptive stalk region is even more intense while the mature stalk and spore cells show little or none.<sup>3</sup> Those cells, then, which are preparing for, or actually in the process of making stalk, are the ones which have large amounts of the enzyme.

#### CONCLUSIONS PERTINENT TO *D. DISCOIDEUM*

From a morphogenetic point of view the polysaccharides are of prime importance: they are the supporting substances

<sup>3</sup> Occasionally, during culmination, a small wedge of intense alkaline phosphatase activity is evident about the base of the spore mass and these cells also show the characteristic stalk polysaccharide distribution, as well as intense staining with Nile blue sulphate in the living organism. Since these cells are also often found in the posterior end of the migrating sausage, it is not clear if they are stragglers or if they are a well defined zone of cells important in the morphogenetic processes.

in the smooth elliptical capsule of the spores, the tapering cylinder of the stalk and probably the slime sheath itself. It is not surprising, therefore, that a study of this key class of chemical substances reveals and even anticipates the course of the morphological changes that unfold during differentiation.



Fig. 5 Photographs of sections prepared to reveal the distribution of alkaline phosphatase. *a*, late migration stage; *b*, the beginning of culmination. Note that in both cases the presumptive stalk region shows a high concentration of the enzyme.

Of these changes, the transition from amoeba to spore seems to be a very conservative one in the sense that the volume changes (as revealed by the measurement of the nucleus) show a smooth, continuous diminishing sweep, the PAS reaction becomes progressively more intense, and there is virtually no demonstrable alkaline phosphatase activity. On the other hand, the conversion of amoebae to stalk cells is a progressive, active process: the cells reverse the tendency to decrease in size and start to swell; the PAS reaction fades



and then there is a period of active secretion of the stalk wall, the cells secreting as though they were a glandular epithelium. Furthermore, the cells of this anterior region are high in alkaline phosphatase indicating some energy-involved activity; and finally, let it not be forgotten that this is the region that emits most and then, at the end, all the acrasin (Bonner, '49).<sup>4</sup>

In a sense the anterior cells are responsible for the shape of the organism. They produce the stalk and, at least during the later stages of culmination, all of the slime sheath. They are the busy doers and the miserly spore cells seem to ride along, expending no energy, giving nothing out, keeping all within the tight locket of the spore case ready for the next generation. Raper ('40) showed sometime ago that if a migrating sausage is cut into segments the posterior pieces made of presumptive spore cells only will soon produce stalk cells; it is no problem for the presumptive spores which have made no changes and kept everything to make the necessary transition to a stalk cell. But, on the other hand, an anterior segment made up of presumptive stalk takes a long time to revert and produce the normal number of spore cells. For a cell which has started its reckless way of becoming part of a stalk, any backtracking or changing of its fate is a slow and difficult process, not to be considered lightly.

Now we will turn to other species of the Acrasiales and compare their polysaccharide and alkaline phosphatase patterns with that of *D. discoideum*.

<sup>4</sup>Since this was written the recent work of Gregg, Hackney and Krivanek ('54) has appeared and their determination of nitrogen changes during the development of *D. discoideum* fits in well with the above results for they find that there is an appreciable loss of protein nitrogen (per dry weight) during culmination, and this loss is largely in the stalk cells. Also in an abstract Gregg and Bronsweig ('54) report that there is a total increase in the reducing substances (assumed to be primarily reducing carbohydrates) during development and possibly an especially significant increase in the stalk region. Since there is no intake of food during development, these authors propose that nitrogenous substances solely are being metabolized and from this energy is being provided to synthesize carbohydrates. Furthermore, they suggest that this process is taking place principally in the active presumptive stalk region.

*D. mucoroides* and *D. purpureum*. These two species differ from *D. discoideum* in that they do not have a stalkless migration period, but rather the stalk is formed during the latter part of aggregation, and the culminating mass usually lies close to the substratum as though it were migrating and forming a stalk simultaneously, finally rising upward to form a mature fruiting structure basically similar to *D. discoideum* except for the total shape of the stalk and the absence of the basal disc (fig. 6). *D. purpureum* differs from *D. mucoroides* primarily in the fact that its final spores have a deep purple color, as its name implies. They are discussed here together because except for one difference which will be examined presently, they are essentially similar in their polysaccharide and alkaline phosphatase distribution.

In both these species, besides the fact that the stalk forms towards the end of aggregation, it is possible to find at this stage a sizeable mass of cells that is already partially differentiated. The stalk which is also formed at the tip shows the same pattern of deposition of polysaccharide as in *D. discoideum* and the presumptive stalk cells, which comprise a remarkably small zone are also, when prepared by the PAS method blanchied in their appearance as in *D. discoideum*. The large mass of presumptive spore cells is dark, but in the early stages it shows, in *D. mucoroides*, a clear gradient by being especially dark with deeply stained, small, uniform granules in the cells of the anterior end and the color diminishing in intensity until the amoebae resemble totally undifferentiated cells at the posterior end. In *D. purpureum* the presumptive spore mass more closely resembles *D. discoideum* and is uniformly stained.

In both *D. mucoroides* and *D. purpureum* the anterior cells of the presumptive spore area become progressively converted into presumptive stalk cells; that is, the sharp division line moves posteriorly during development. This was demonstrated by making camera lucida drawings of longitudinal sections at the beginning, middle and towards the end of culmination. By the use of a planimeter the areas of the

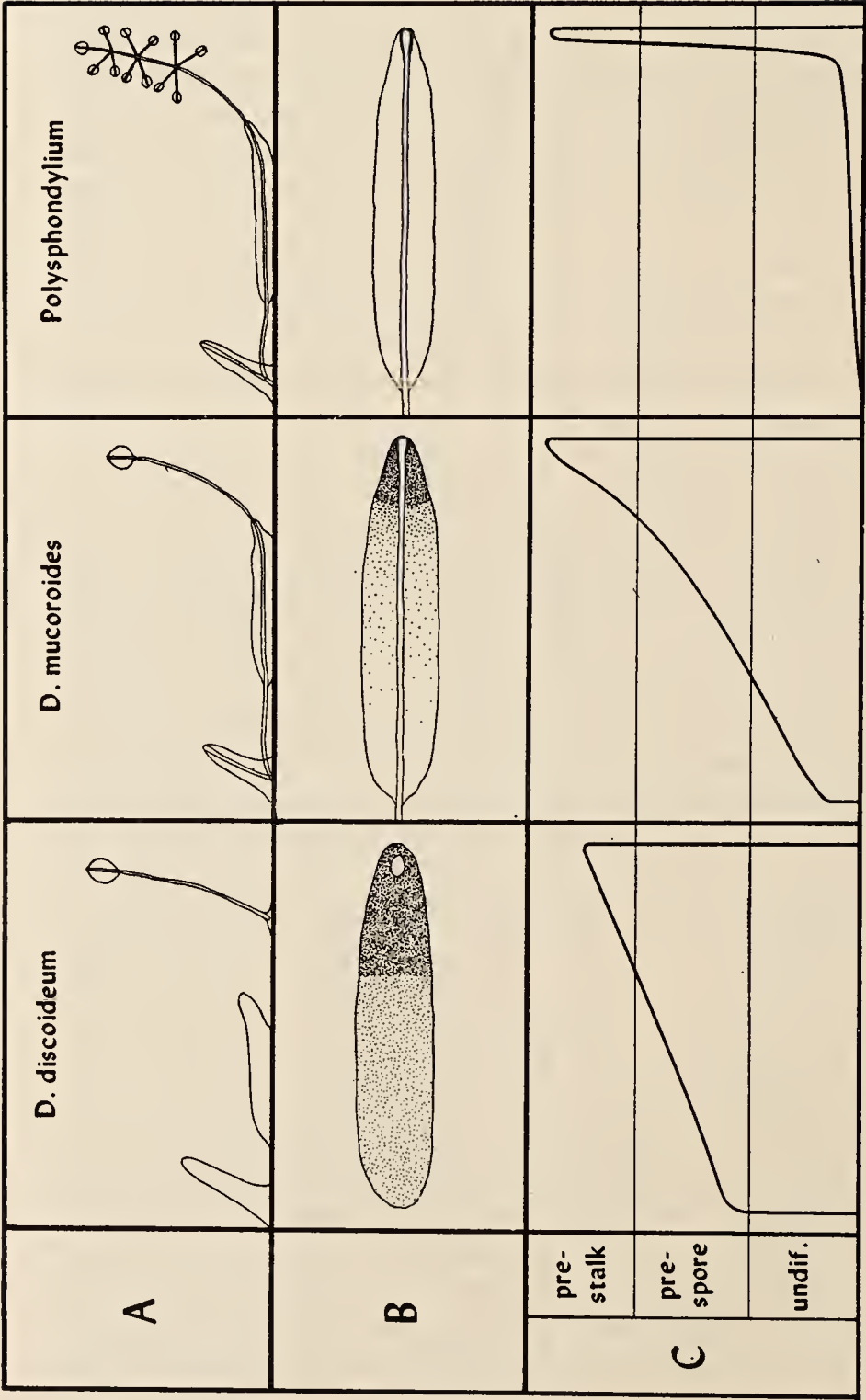


Fig. 6 A series of diagrams comparing various species of Acrasiales with respect to (a) their morphological characters; (b) their staining characteristics during late migration (or an equivalent stage) as revealed by the PAS and the alkaline phosphatase techniques; (c) their hypothetical level of differentiation (see fig. 7) along the axis of the cell mass at the stage indicated immediately above in B.



presumptive spore and stalk regions of a medial section could be compared. In 6 cases with *D. mucoroides* and 4 cases with *D. purpureum* it was shown that the per cent presumptive stalk area actually increases as development proceeds, showing that presumptive spores must have been converted to presumptive stalk cells. This is an entirely different situation from *D. discoideum* where the division line is fixed (at least until the beginning of culmination) once it is laid down during the migration stage.

As far as the alkaline phosphatase is concerned, it again showed high activity in the presumptive stalk region so that there was a conspicuous black collar about the tip of the stalk.

*Polysphondylium violaceum* and *P. pallidum*. The last two species used were of the genus *Polysphondylium* which, like *D. mucoroides* and *D. purpureum* form a stalk directly following aggregation, but differ from them in that during the final culmination clumps of cells are detached on the stalk at regular intervals and each of these forms a whorl of usually 2 to 4 small fruiting bodies that jut off at right angles from the parent stem (fig. 6a). *P. pallidum* has white spores, while the spores of *P. violaceum* are purple, similar to those of *D. purpureum*.

Again with the polysaccharide method the stalk showed the typical pattern of deposition right from the end of aggregation, but throughout the development there is no evidence for any region of presumptive spore or stalk cells. All the cells appear undifferentiated until they either become trapped in the stalk tip or they turn into spores. *P. pallidum* showed a slightly more intense coloration throughout the cell mass than *P. violaceum* but in neither case did they show a division line or staining intensity comparable to the presumptive spore cells in *Dictyostelium*. Repeated attempts were made to catch a transition period in spore differentiation, but in every case the spores were either encapsulated and mature, or the cells were quite undifferentiated. If there is an intermediate phase as in other species, it must be extremely ephemeral.

Concerning the alkaline phosphatase, it was impossible to demonstrate any high concentration in any region.

#### GENERAL CONCLUSIONS

In comparing the three types of development described, that of (1) *D. discoideum*, (2) *D. mucoroides* and *D. purpureum*, and (3) *Polysphondylium* we find that not only is their external morphology different, but also they differ in their polysaccharide alterations as well as their pattern of alkaline phosphatase activity. It is possible, in a diagrammatic fashion, to compare the three types, as shown in figure 6A.

If first the sequences of events in stalk formation are compared, then *D. discoideum* form its stalk slowly and late; the early signs of the stalk do not appear until the middle of migration and mature stalk cells are not formed until the beginning of culmination. In *D. mucoroides*, *D. purpureum* and *Polysphondylium* the production of mature stalk cells begins immediately at the end of aggregation.

Concerning the spores, *D. discoideum* shows a relatively rapid formation, for the presumptive spores appear as an even zone in the middle of the migration period and begin to mature soon after the beginning of culmination. In *D. mucoroides* the rate of formation is similar to *D. discoideum* but the details differ. The presumptive spores show a gradient in their degree of differentiation and, furthermore, the presumptive spores near the anterior end are converted into presumptive stalk cells during the long process of stalk formation. *D. purpureum* shows an intermediate situation where the division line moves posteriorly as in *D. mucoroides*, but the presumptive spore area is evenly stained as in *D. discoideum*. In *Polysphondylium* the presumptive spores are very slow to show any signs of differentiation, but when they do at the end of culmination they seem to go extremely rapidly from an undifferentiated state to a mature one.

We can, from the information presented here, make a chart showing all the conversions that normally take place during development in the Acrasiales (fig. 7).

Undifferentiated cells can obviously develop into either partially differentiated spore or stalk cells — this is the normal course of events. Furthermore, in *D. mucoroides* and *D. purpureum* partially differentiated spore cells close to the division line normally are converted to partially differentiated stalk cells. As the small downward pointing arrows indicate all these processes are reversible using various experimental procedures, as has been shown in earlier work (Raper, '40, Bonner, '52). The only steps that are, so far as is known, irreversible are the final differentiations into mature spore and stalk cells, although, in a sense the spores upon germination revert back to the undifferentiated condition.

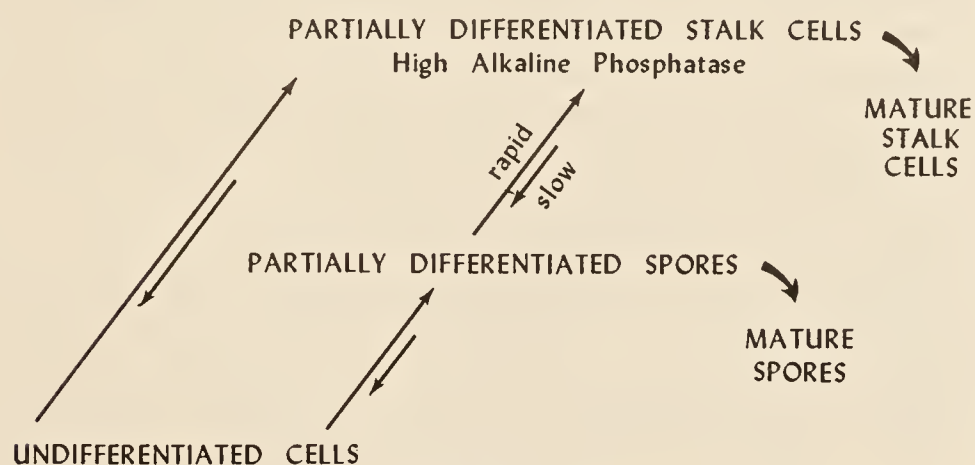


Figure 7

Now let us assume that the direction of differentiation indicated in figure 7, namely, from undifferentiated cells to partially differentiated spore cells to partially differentiated stalk cells, involves three steps in a continuous process. For comparison of the various species let us now take a stage at, or equivalent to, the middle of migration for each and indicate these advancing steps by increasingly dark areas (fig. 6b). Or the same point may be illustrated by three graphs in which the abscissa is the length of the cells mass while the ordinate shows the steps given above (fig. 6C). It is obvious in *Poly-*



*sphondylium* that the gradient is extremely sharp compared to *D. discoideum*. Therefore, the species differ in the rates and the time at which maturity is reached along the axis of the cell mass and it is reasonable to assume that the basis of the overall morphological difference of these forms lies in the kinetics of the differentiation process.

If this hypothesis is correct, then it brings us close to the notions of C. M. Child and R. Goldschmidt concerning rates of processes. Child ('41) has emphasized the importance of activity gradients along the axis of an organism in determining the ultimate development and Goldschmidt ('38) has stressed the genetic control of the rates of processes and how they affect the pattern of the mature individual. It has frequently been pointed out that these theories do not explain all the aspects of development and if such rate processes are involved here it is now imperative to know how they in turn are controlled in the different species and how this control exerts its action along the axis of the cell mass.

There is one interesting corollary to the hypothesis. The posterior portions of *Polyphondylium* remain immature until almost the end of development, and this very fact allows for the breaking up of the cell mass into essentially small, separate, new individuals that form the whorls. If the cells did not remain so undifferentiated for so long a period of time such a complete new development would likely be impossible. This, then, is a case of the prolonging of youthful characters into adult life and in some ways it approaches the phenomenon of neoteny found in some higher organisms.

#### ACKNOWLEDGMENTS

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of Trinity College, Cambridge, for his interest and his help during the course of this work.

#### SUMMARY

A detailed study was made with various histochemical techniques of different stages of development of the cellular slime mold *Dictyostelium discoideum* and it was found that the distribution of two types of substances, the non-starch polysaccharides and the alkaline phosphatases were especially helpful in revealing information concerning the morphogenetic processes. At relatively early stages there are detectable differences between the distribution and staining properties of the polysaccharides of the presumptive spore and stalk cells. In the spores the cells progressively decrease in size and the polysaccharide is partly kept in the cytoplasm and partly incorporated into the spore casing. In the stalk cells the polysaccharide is largely exuded in a specific zone of stalk deposition by the presumptive stalk cells as they rise to the tip during culmination, and the polysaccharide that remains in the mature stalk cells can be seen in the cytoplasm, and the cell wall. The presumptive stalk cells alone show high alkaline phosphatase activity which is not inconsistent with the notion that spore formation is a relatively passive process while stalk formation is the important morphogenetic activity involving a high turnover of energy.

In comparing *D. discoideum* with other species, namely *D. mucoroides*, *D. purpureum*, *Polysphondylium violaceum* and *P. pallidum*, it was shown that there were at least three characteristic and different polysaccharide and alkaline phosphatase patterns. By assuming that these differences represent differences in rates (and timing) of the differentiation process and by assuming a definite sequence of the steps of differentiation, it is possible to interpret the morphological differences of the various species in terms of the rates and the time at which maturity is reached along the axis of the cell mass.

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## A THEORY OF THE CONTROL OF DIFFERENTIATION IN THE CELLULAR SLIME MOLDS

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IN THE life history of cellular slime molds separate independent amoebae aggregate in masses, and each of these masses proceeds to develop as a unified individual. Some of the amoebae within the mass differentiate into stalk cells, and the remainder differentiate into spores. Depending upon how many amoebae enter the aggregate, there is a wide variation in the size of the fruiting bodies. Yet there is a constant relationship between the number of stalk cells and the number of spore cells. This problem of proportional development is of basic importance not only in the slime molds but in all organisms exhibiting regulative development.

My colleagues and I have ascertained many pertinent facts on this proportionality of development in the slime molds in earlier work, and some new original information will also be included here. From this whole array it is possible to construct a theory which ties these facts together. There is no assurance that the theory represents the actual mechanism of development, and certainly alternative theories could be proposed that would fit the facts. This particular theory, however, has the merit of simplicity, and does serve to illustrate what kind of a mechanism could conceivably be operating in the development of these organisms.

Before describing the theory it will be well to review some of the known facts, and to present some original material that will serve as a basis for the theory. But since there may be some readers who are not completely familiar with the cellular slime molds, I shall first discuss briefly the life history of *Dictyostelium discoideum* and then compare it with some related species.

The spores of *D. discoideum* are covered with a hardwalled capsule. Upon germination each capsule liberates one amoeba. The amoebae divide mitotically and remain entirely separate from one another, feeding independently upon bacteria. When they multiply to a population of sufficient

numbers, they stream together to form large collections of many cells or pseudoplasmodia, and this aggregation process appears to be largely due to a chemical substance, acrasin, to which the amoebae are chemotactically sensitive. Certain of the amoebae, which form the center of the aggregate, apparently produce it sooner than others, and in this way an acrasin gradient is set up which is effective in orienting the amoebae.

The aggregated cell mass assumes a sausage shape and crawls about the substratum for variable periods of time. During this migration phase it is sensitive to light and heat gradients, orienting itself toward light and toward warmer regions. Differentiation begins at this stage. The anterior cells of the sausage are destined to become part of the supporting stalk and the posterior cells will turn into spores. The final fruiting involves a series of morphogenetic movements in which the anterior presumptive stalk cells are pushed down through the spore mass, and in so doing, these stalk cells become large and vacuolate and are permanently trapped in a delicately tapering cellulose cylinder. During this culmination stage, the spore mass is lifted up into the air and each amoeba in the mass becomes encapsulated into a spore (Fig. 1).

In *Dictyostelium mucoroides* and *D. purpureum* there is a basically similar development, except for the fact that during the migration period the stalk is formed continuously. Its formation begins right after aggregation, and as the cell mass crawls across the substratum, again strongly oriented towards the light and heat, the stalk is constantly deposited at the tip (Fig. 1). The only exception to this, as Raper and Thom (1941) have found, is that in certain strains there are periodic discontinuities in the stalk, which indicate occasional intervals of migration without stalk formation.

The genus *Polysphondylium* represents another type of development which bears many similarities

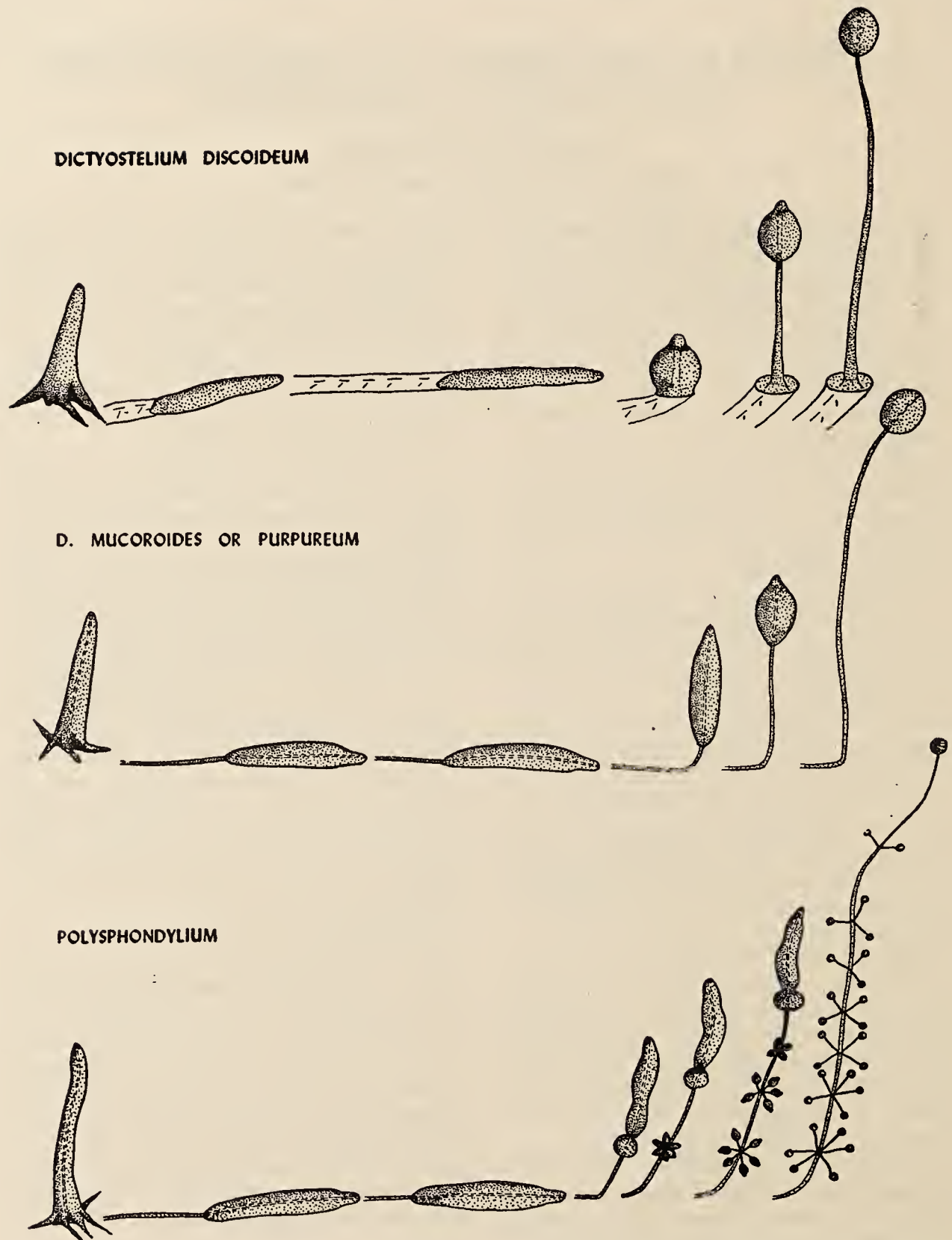


FIG. 1. THE MIGRATION AND CULMINATION STAGES IN THREE DIFFERENT TYPES OF CELLULAR SLIME MOLDS

to the *D. mucoroides* type, but differs in the pattern of the final fruiting. After a period of migration, while again the stalk is being continuously constructed, the cell mass breaks into fragments in the posterior portion, leaving behind a series of cellular collars about the stalk that cease all forward movement. Each of these dough-nut-shaped cell masses breaks up into several



radially oriented secondary fruiting bodies, so as to give the appearance of a series of whorls arising at fairly regular intervals, with one large *D. mucoroides*-like sorus at the tip (Fig. 1).

The theory to be proposed shortly is designed to explain certain aspects of this remarkable kind of development found in the cellular slime molds. The best way to expose the problem to be illuminated is to frame three questions, and in attempting to answer each of these questions some understanding of a possible mechanism of the control of differentiation can be obtained. The three questions will be stated briefly first, and then each one will be examined in detail.

(1) One of the basic facts is that there is a differentiation in which two cell types are produced, the stalk cells and the spore cells. As previously mentioned, the stalk cells are produced at the anterior end of the cell mass, and the spore cells at the posterior end. So the first question to ask is: *what are the differences between the front and the hind ends of the mass?*

(2) There occurs normally in these molds a great variation in size of the cell mass. Since feeding ceases before aggregation, the total size of the aggregate depends upon the quantity of amoebae that enter into it, and large fruiting bodies may in extreme cases be a thousandfold larger in volume than small ones. Nevertheless, the size of the spore mass bears a constant, proportional relationship to the size of the stalk, and our second question is: *how, in any one species, is it possible that the differentiation remains proportionate irrespective of size?*

(3) In recent studies we have shown that different species (and even different strains of one species) have different proportionality relationships. Therefore the third question is: *how can one account for differences in proportionality ratios in different species and strains?*

#### THE DIFFERENCES BETWEEN THE FRONT AND HIND ENDS OF THE MASS

The facts relevant here have mostly been presented previously, and it is necessary only to summarize and bring out some of the pertinent points. For simplicity, the situation in *D. discoideum* will be used as the main example, and the variations of the other species will be added later.

In the first place, it was found that if all the amoebae were stained with a vital dye such as Nile blue sulfate, Bismarck brown, or neutral red

before aggregation, then after aggregation the migrating cell mass would appear uniformly stained. At a later period, however, the anterior, presumptive stalk end remained dark, while the posterior, presumptive spore portion blanched (Bonner, 1952). The division line between these two zones remained sharp and distinct as the migrating mass crawled over the surface of the substratum. The limitation of these experiments is that while they provided an excellent tool for revealing vitally the extent of the early presumptive stalk and spore areas, they give little indication as to what might be the chemical and physical basis of such changes.

Another approach was to measure cell and nuclear sizes (Bonner and Frascella, 1953; Bonner, Chiquoine, and Kolderie, 1955). Both measurements were consistent in that they showed that during a period where a division line was evident after using vital dyes, the anterior cells and their nuclei were larger than those of the posterior portion. In the studies on nuclear size it was shown that this difference was only evident after a period of migration, for very young migrating cell masses showed no differences between the nuclei of the front and hind ends.

The most illuminating studies of the difference between these two regions were those performed using histochemical techniques in collaboration with Dr. Duncan Chiquoine of Princeton University. In particular, the examination of the distribution of non-starch polysaccharides showed that at the onset of migration the cell mass was evenly stained, but that after a period of crawling the posterior cells contained small, dense, intensely stained granules of non-starch polysaccharides, while the anterior cells were, by comparison, pale and diffuse in their staining reaction (Fig. 2). However, it was clear from sections of the culmination stage that the anterior presumptive stalk cells did contain non-starch polysaccharides which accumulated in their centripetal ends as they advanced into the tip of the cell mass. Their orientation is radial, and obviously they are secreting the cellulose cylinder. When largely depleted, they arrive at the tip and become incorporated into the stalk, the cellulose cylinder that they themselves have manufactured (Bonner, Chiquoine, and Kolderie, 1955). Once in the stalk they progressively swell and become large and vacuolate.

In the same histochemical study we examined



the distribution of alkaline phosphatase. In late migration the anterior presumptive stalk cells were clearly high in alkaline phosphatase, and in the culmination stage this difference was even more sharply defined. However, little or no activity was shown by the cells once they had entered the stalk.

So in answer to our question of what are the differences between the front and hind ends, we can say first off that there are numerous demonstrable differences, and undoubtedly many which have yet to be revealed. But the mere listing of differences is in itself of limited interest; it is desirable to attempt to attribute some meaning to these differences.

Consider a fruiting body in its mature form. It is obvious that since it sticks up into the air it is dependent upon the rigid cellulose cylinder of the stalk. The basis of its form, its skeletal support, comes from the production (and proper distribution) of polysaccharide. The anterior cells, then, are the active cells as far as the morphogenesis of this organism is concerned; they are the ones responsible for the final form. It is impossible at our present state of knowledge to ascribe any specific task to the presence of high alkaline phosphatase, but again it is consistent with the notion presented here that the front end is active and vital in development. This idea is also supported by the work of Gregg, Hackney, and Krivanek, (1954) who found that there is an appreciable loss of protein nitrogen during culmination (presumably used as an energy source for carbohydrate synthesis), and that this loss occurs only in the stalk region.

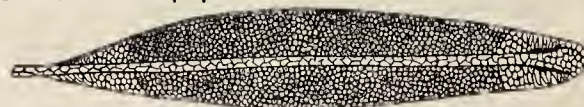
The activity of the anterior pre-stalk cells is indeed striking, and yet in the final fruiting body the mature stalk cells are mostly trapped and dead inside the stalk cylinder. From these twin facts it is hard to avoid the conclusion that the stalk cells have thrown themselves into their morphogenetic activities with such energy that they have lost the ability to perpetuate themselves, and that instead they become depleted, exhausted, inert bricks that fill up the inside of the stalk.

The presumptive spores, on the other hand, appear to give off nothing; they neither show a high enzymatic activity (viz., of alkaline phosphatase), nor are they wasteful of their polysaccharides. They would seem to be conserving all for the next generation and they make little or no contribution to our immediate understanding of

*Dictyostellium discoideum*



*D. mucoroides* or *purpureum*



*Polysphondylium*



FIG. 2. THE STAINING PROPERTIES OF THE CELLS DURING THE MIGRATION PHASE FOR THREE DIFFERENT TYPES OF CELLULAR SLIME MOLDS

morphogenesis. When one considers the activity of these two cell types, it is indeed a striking division of labor.

In turning to comparative studies of other species, the *D. mucoroides* type (which includes *D. purpureum*) shows much the same pattern except for the fact that the presumptive stalk region appears consistently smaller. The significance of this point will be discussed later (Fig. 2). *Polysphondylium*, on the other hand, shows none of these early signs of differentiation; both the front and hind cells appear, even after considerable migration, to be evenly stained by the methods previously mentioned. Differentiation occurs very suddenly at the end of its cycle, after the doughnut-shaped rings of cells have been cut off and left behind. As pointed out previously (Bonner, Chiquoine, and Kolderie, 1955), this prolongation of youthful, embryonic characters until the last moment may be correlated with the ability of *Polysphondylium* to fragment into small fruiting bodies after a period of migration. But even in this case the sequence of events in differentiation must be basically the same, the only unusual aspect being its timing mechanism.

In conclusion, it should be emphasized that the principal difference between the front and hind ends is one of activity. There are reactions taking place in the front end which are crucial to the morphogenesis of the organism and which have no counterpart in the hind end. There are, of

course, still many questions one would like to ask about these reactions. It would be desirable to have a fuller understanding of the chemical changes involved; for instance, to learn if there is any relation between this anterior activity and the fact that the front end is always the region of high acrasin emission (Bonner, 1949). But now that we have underlined some of the major antero-posterior differences we can attack the problem of proportionality.

HOW, IN ANY ONE SPECIES, IS IT POSSIBLE THAT  
DIFFERENTIATION REMAINS PROPORTIONATE  
IRRESPECTIVE OF SIZE?

The first thing that must be done is to give the evidence that proportionality does hold in the slime molds. A superficial examination of a culture of *D. discoideum* under the dissecting microscope makes one strongly suspect that such is the case, for both the small and large fruiting bodies will have a similar appearance. This was pointed out by Raper (1935) in his original description of the species. If one takes the pains to measure these mature fruiting bodies accurately, as we did some years ago (Bonner and Slifkin, 1949), then it is clear that with increase in size the sorus volume is roughly linearly proportional to the volume of the mature stalk.

Recently (in collaboration with Miss Marcia J. Shaw) we have studied the proportions of the anterior presumptive stalk cells and the posterior presumptive spore cells in the migrating stage. Of necessity we had to use different techniques for the different species. The most desirable method, which would work only with *D. mucoroides* and *D. purpureum*, was to make fixed and stained whole mounts of the cell masses, using a methylene blue technique developed by Mr. Vincent R. Gregg, Sr., of this laboratory (See Appendix). From camera lucida drawings of the migrating masses, their volumes were calculated by approximating each part to the form of a cylinder. With *D. discoideum* the method of measurement was the same, but the camera lucida drawings were made of live cell masses stained vitally with Nile blue sulfate (Bonner, 1952).

As one can see from Fig. 3, there is a straight line relationship if, for *D. discoideum* and *D. mucoroides*, the volume of the presumptive stalk region is plotted logarithmically against the volume of the presumptive spore mass or sorus. To avoid anticipating the next question, I will

not compare the two species at the moment, but rather would like to point out that in each case there is proportionality, and that in *D. mucoroides* especially it extends over a considerable size range. This means, then, that not only is the final differentiated product proportional, but so are the presumptive areas as well.

One of the most interesting aspects of proportionality is to be seen in the phenomenon of regulation. This was beautifully illustrated in a pioneer experiment of Raper (1940) in which he cut migrating pseudoplasmodia of *D. discoideum* into fractions and noticed that each fraction gave a normal fruiting body, although, this took some additional time to be achieved in the anterior portion. (Fig. 4).

This experiment has been repeated a number of times, both using the vital dye method (Bonner, 1952) and using the method which stains specifically for the non-starch polysaccharides (Bonner, Chiquoine, and Kolderie, 1955). From these studies it is possible to show that the staining properties so characteristic of the anterior and posterior regions are actually reversible. In Fig. 5 one can see that one hour after the all-dark posterior portion of presumptive spore cells has been isolated, there begins to appear a small pre-stalk area at its tip, and by six hours a small but normally proportional cell mass is evident. The converse is true of the isolated tip, inasmuch as it shows a progressive appearance of presumptive spore cells at its posterior end. Clearly, in both cases there has been a reversal of the fate of the cells (which follows Driesch's dictum in that they are determined by their position in the whole) as well as of their staining properties.

Fig. 3, which shows the proportionality of the presumptive spore and stalk areas in the two species of *Dictyostelium*, reveals an interesting point which concerns regulation. Methods have been devised to encourage extensive, long migration in both *D. discoideum* (Slifkin and Bonner, 1952) and *D. mucoroides* (Bonner and Shaw, in press). In both these species, when the methods previously mentioned are used, it is possible to check the proportions at different points during an extended migration up to 70 mm. in length. In neither species did the proportions show any alteration after prolonged migration. In all cases the points remained on the lines indicated in Fig. 3. This is especially significant in the case of *D. mucoroides*, for in that species, as migration



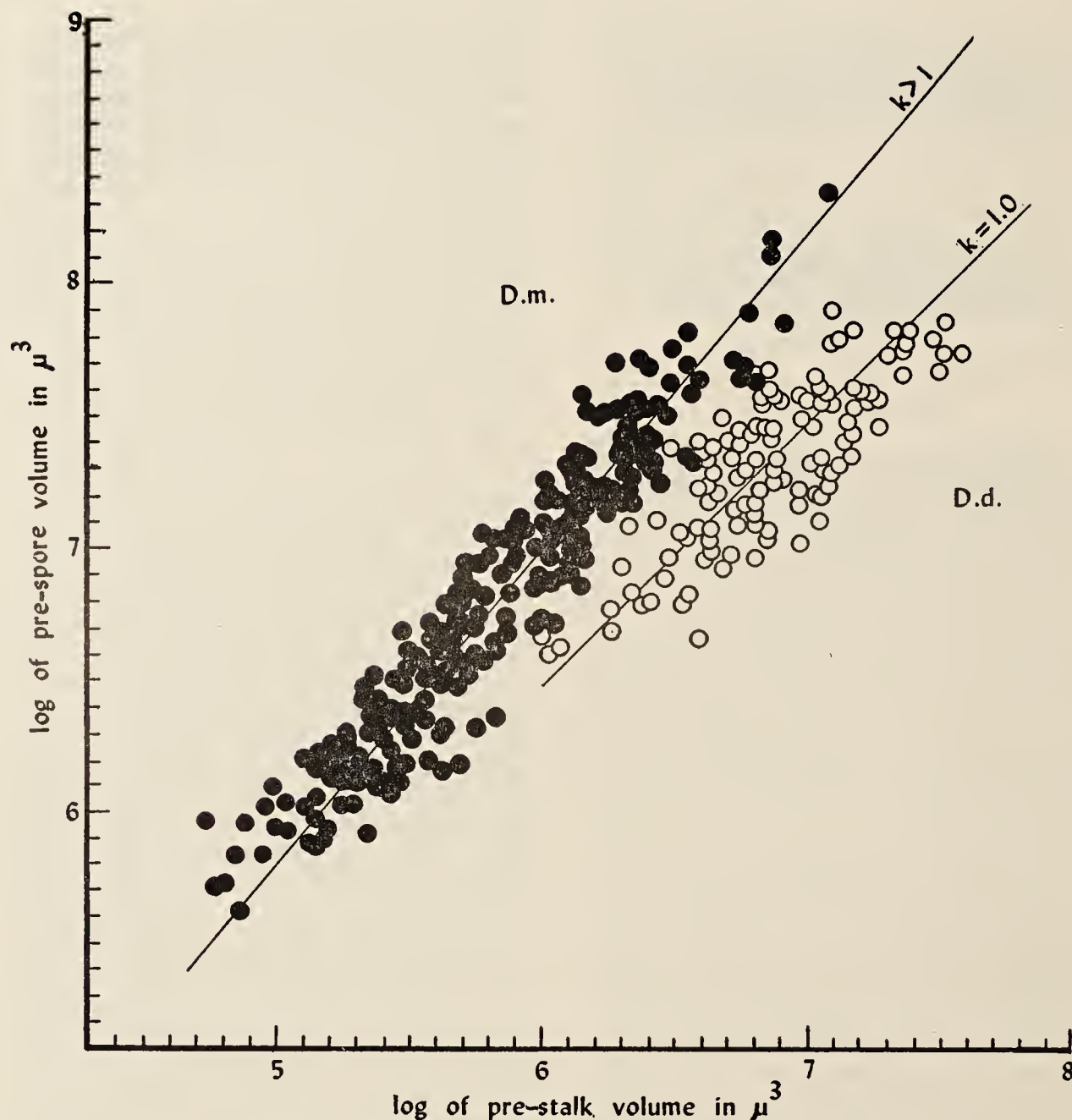


FIG. 3. PRE-SPORE VOLUME PLOTTED LOGARITHMICALLY AGAINST PRE-STALK VOLUME FOR *D. MUCOROIDES* AND *D. DISCOIDEUM*

The slopes of the lines ( $k$ ) are roughly indicated. The solid dots actually include 6 strains of *D. mucoroides* tested, all of which were similar, as were 3 strains of *D. purpureum* which are not included. The only exception is DM-4, which gave points parallel to, but above the other *D. mucoroides* strains. See text for details.

continues, there is a constant loss of cells into the stalk, since this structure is being formed at all times. In other words, there is a continuous disappearance of cells at the tip, but since the division line shows a fixed proportionality, it must be constantly regulating, by moving posteriorly. If a cell mass is large in the beginning, its proportions will be such that it will lie on the upper end of the curve for *D. mucoroides* in Fig. 3. With

extended migration (up to 220 mm. in some cases), the mass will become smaller and move down the curve so that when it finally fruits it will have a gigantic stalk 220 mm. long and a minute sorus, although at any point in its journey the presumptive areas will show proportionality.

At this point a hypothesis may be proposed which is perhaps the most important element in the whole theory. To account for proportionality



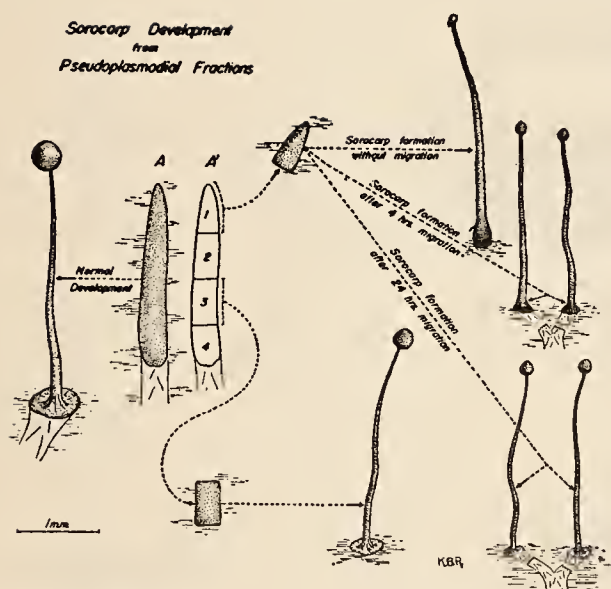


FIG. 4. COMPARISON OF THE FRUITING OF ENTIRE CELL MASSES WITH DIFFERENT FRACTIONS OF THE SAME

If apical fractions fruit immediately they show abnormal proportions, but with some migration the normal proportions are resumed (from Raper, 1940).

and regulation it must be assumed that one part of the cell mass "knows" the extent of another. When a pre-stalk tip is isolated, the information that all the posterior pre-spore cells are missing is somehow registered, so that in a matter of a few hours the deficit is made up and regulation has taken place. A logical conclusion is that there must be some communication between parts. Let us therefore postulate a polar movement of some key factor which provides the necessary information. In our previous example, if the posterior portion is removed, so also is a large source of the polar factor, and consequently regulation ensues. If the anterior portion is removed, then although there may still be an appreciable supply of polar factor in the posterior portion, the anterior reaction which is dependent upon the polar factor must start and establish itself at the new tip. An equilibrium, i.e., a balance between the movement and the reaction, will soon result. The nature of this balance will be discussed shortly, but at the moment two things in this hypothesis need further clarification: the nature of the factor, and the way in which it is transported in a polar fashion.

A number of observations make it very tempting to suggest that the polar factor can be transported by especially fast-moving cells, which in essence serve as polar messengers. It is an old observation

that the cells do not keep a rigid relation one to another during migration, but that their speeds are variable and independent (Bonner, 1952). Now a series of experiments was done in which a posterior half of a migrating cell mass of *D. discoideum* stained vitally with Nile blue sulfate or neutral red was grafted onto a colorless anterior half, and the extent of the dye was then followed for a number of hours. It could be seen with the dissecting microscope that a number of intensely stained cells would form a front echelon and stream towards the anterior tip, so that within approximately six hours these fast cells would cover half the length of the actively migrating mass (Fig. 6). That this is the result of some cells being especially rapid in movement and not simply a consequence of diffusion is supported by a previous experiment, one in which an anterior, vitally stained group of cells was grafted into an intact, colorless migrating mass. In a matter of approximately three hours these cells moved to the tip, but in this case the final result was not an all-colored cell mass, as in the first case, but instead the colored cells moved up as a band (Bonner, 1952). From this we may conclude that the anterior cells are fast-moving and the posterior ones are slow.

If a colorless posterior portion is grafted onto a vitally dyed anterior portion the color will move backward at approximately the same rate as the forward movement just described. Therefore, as one would expect in a variable population of cells, there are not only especially fast cells but especially slow cells as well. We have some further interesting information about the slow cells: in *D. mucoroides* they apparently accumulate in the posterior end of the migrating mass so that if migration occurs over an extended period, then there is a steady increase in the accumulation of slow cells (Fig. 7). It is possible to discern these cells both in the total mounts when using Gregg's methylene blue technique and also in the technique for the non-starch polysaccharides (see Fig. 2). In fact, it is evident from the latter procedure that these accumulating slow cells resemble the anterior presumptive stalk cells, for they are large in size and show the typical anterior staining properties (Bonner, Chiquoine, and Kolderie, 1955). It is possible that these cells have participated in the anterior, energy-consuming reaction, but have escaped becoming incorporated into the stalk, and that now, since they are low in every resource, they

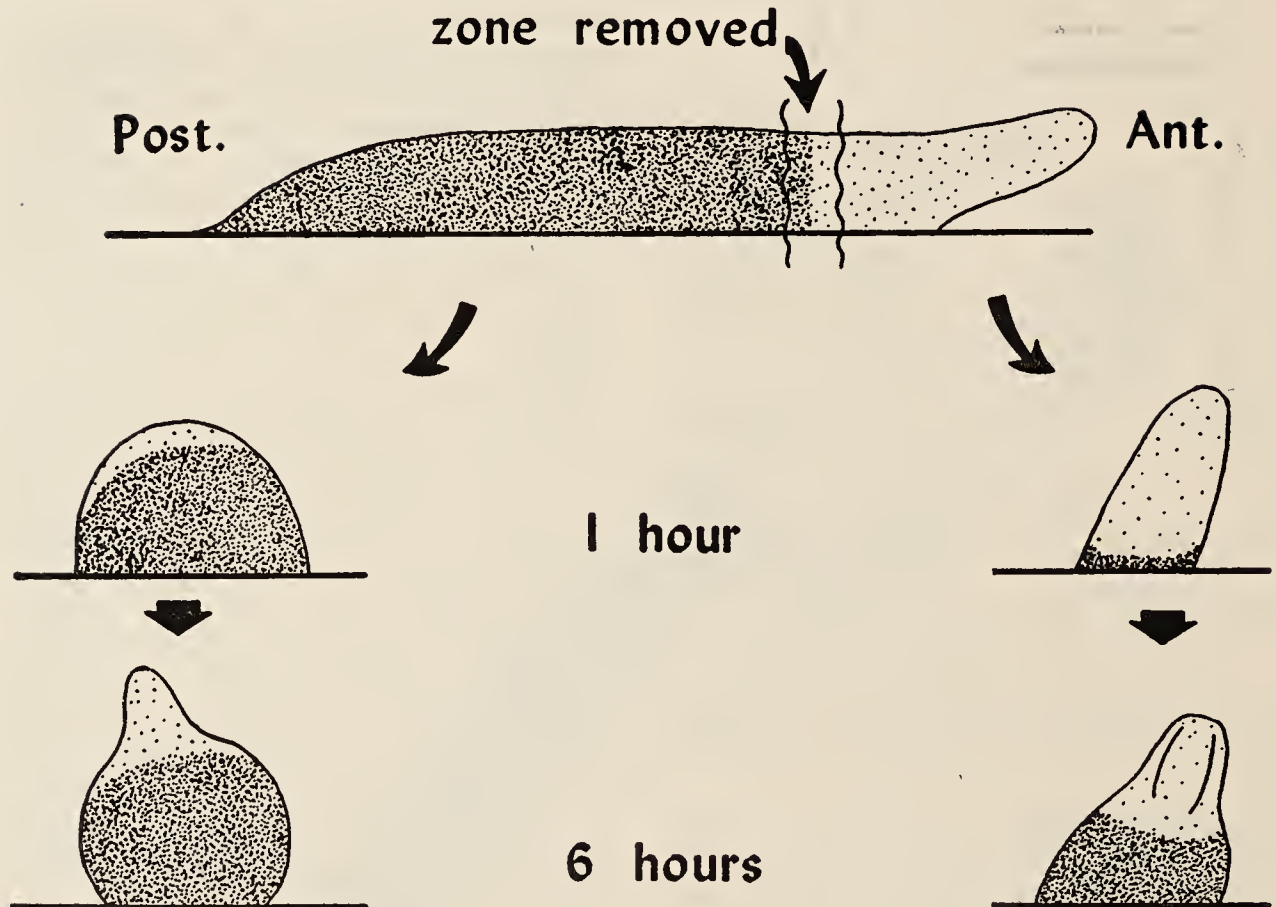


FIG. 5. A DIAGRAM ILLUSTRATING THE EXPERIMENT IN WHICH A PARTIALLY DIFFERENTIATED MIGRATING CELL MASS IS BISECTED AND EACH PORTION IS EXAMINED BY THE PERIODIC ACID—SCHIFF METHOD AFTER 1 AND 6 HOURS RESPECTIVELY

Note that the anterior end of each fragment reversed its staining properties; in one case from the light pre-stalk condition to the dark pre-spore condition, and vice versa in the other (from Bonner, Chiquoine, and Kolderie, 1955).

drop back to become laggards. However, these slow cells do maintain the ability to regulate, and therefore they cannot be too low in energy. It was possible in a number of cases of *D. mucoroides* showing prolonged migration to cut off these cells, and in every instance they gave rise to normal fruiting bodies.

The fact that there is such a group of cells following the pre-spore mass during culmination in *D. discoideum* is a familiar observation (Bonner, 1944; Raper and Fennell, 1952). Furthermore, in the sectioned material of migrating pseudoplasmodia we have made recently, it is possible to observe a small zone of cells having pre-stalk characteristics, and closely resembling the situation observed in *D. mucoroides*. It is interesting that this group of laggard cells would account for the basal disc, a distinguishing feature of *D. discoideum* and the one from which it derives its name. The idea is that this group, which has pre-stalk charac-

ters and which is located posteriorly, will naturally surround the base of the stalk as culmination proceeds; furthermore, its cells will ultimately become large and vacuolate, following the same course of cellular differentiation as the stalk cells proper.

However, if this were so, one might expect that with prolonged migration the basal disc would become excessively large, which is not the case. Its size is clearly independent of the duration of migration. The answer to this paradox is found in the well-known fact that during migration there is a constant loss of cells deposited singly and in small groups in the collapsed slime sheath left behind. This loss will largely account for the fact that upon prolonged migration the cell masses become continuously smaller (Bonner, Koontz, and Paton, 1953). We have recently measured this cell loss by counting the cells along a slime track and found that there is considerable varia-



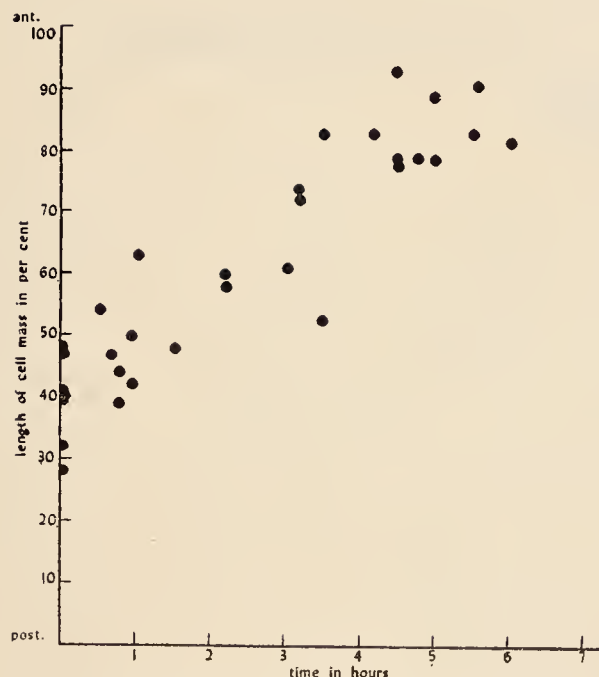


FIG. 6. THE SPEED OF FORWARD MOVEMENT OF CELLS STAINED WITH VITAL DYES IN MIGRATING PSEUDOPLASMODIA OF *D. DISCOIDEUM*

The ordinate shows the position of the marked cells after a colored posterior portion has been grafted onto a colorless anterior portion. The abscissa is time, and it is clear that after six hours the fastest colored cells have reached the tip region.

tion among individual pseudoplasmodia. The rate of cell loss may be high or low at first, but after a migration of 25 mm. the rates rise or fall to a fairly uniform, low rate of approximately 5 to 10 cells per mm. of migration, and then it rises steadily so that by 60 mm. of migration the rate of loss may be anywhere from 10 to 30 cells per mm.

To return to the matter of the fast cells which are possibly bringing the polar factor to the anterior zone, two further points should be added. One is simply that there is no need to postulate any special substance or substances for the polar factor. It could be a simple matter of food or some substrate necessary for a particular enzyme reaction. The characteristic of the front end is activity, and for this energy is necessary. The speed of the cells would imply an energy-rich condition, all of which would fit together. It is of course also conceivable that the cells move anteriorly in an acrasin gradient and that the substrate may contribute to the continued high production of acrasin at the anterior end, but unfortunately the connection with acrasin is especially speculative.

The other point to be made concerning the

polar factor is that there is some evidence to support the contention that the amount of the factor produced depends on the total number of cells or the total volume. If short and fat migrating masses of *D. mucoroides* are compared with extremely thin and long ones, they will appear to have different proportions if the linear distance of the pre-spore and pre-stalk zones are used, but if they are calculated on the basis of volumes they represent a harmonious group (Fig. 8). In other words, the notion that a certain percentage of the total population of cells are fast cells is supported.

To summarize the reply to this second question, an attempt has been made to provide a rational hypothesis which accounts for proportionality and the related phenomenon of regulation. It is suggested that there is a polar movement of substances which provides a communication between parts by delivering these essential substances to the anterior end, where there are reactions responsible for the morphogenesis of the slime mold. Furthermore, it is suggested that this polar movement is achieved by especially fast-moving cells, although it must be remembered that proportional development is also possible in minute fruiting bodies possessing as few as 12 cells (Sussman and Sussman, 1956). In such small pseudoplasmodia there is perhaps no transport, but rather a simple depletion of the factor that is already present in the two anterior cells that make up the stalk.

#### HOW CAN ONE ACCOUNT FOR DIFFERENCES IN PROPORTIONALITY RATIOS IN DIFFERENT SPECIES AND STRAINS?

In Fig. 3 the volume of the pre-stalk area is plotted logarithmically against the volume of the pre-spore area, and each of the two species has a curve that approximates a straight line. The slope and the position of the two differ, however. Furthermore, there is a particular strain of *D. mucoroides* (DM-4) whose points are parallel to, but not coincident with, other strains of *D. mucoroides*, which means that there are three known proportionality relationships.

A straight line on such a logarithmic plot indicates a so-called allometric relation (Huxley, 1932) and can be written in the form

$$y = bx^k$$

or,

$$\log y = \log b + k \log x$$



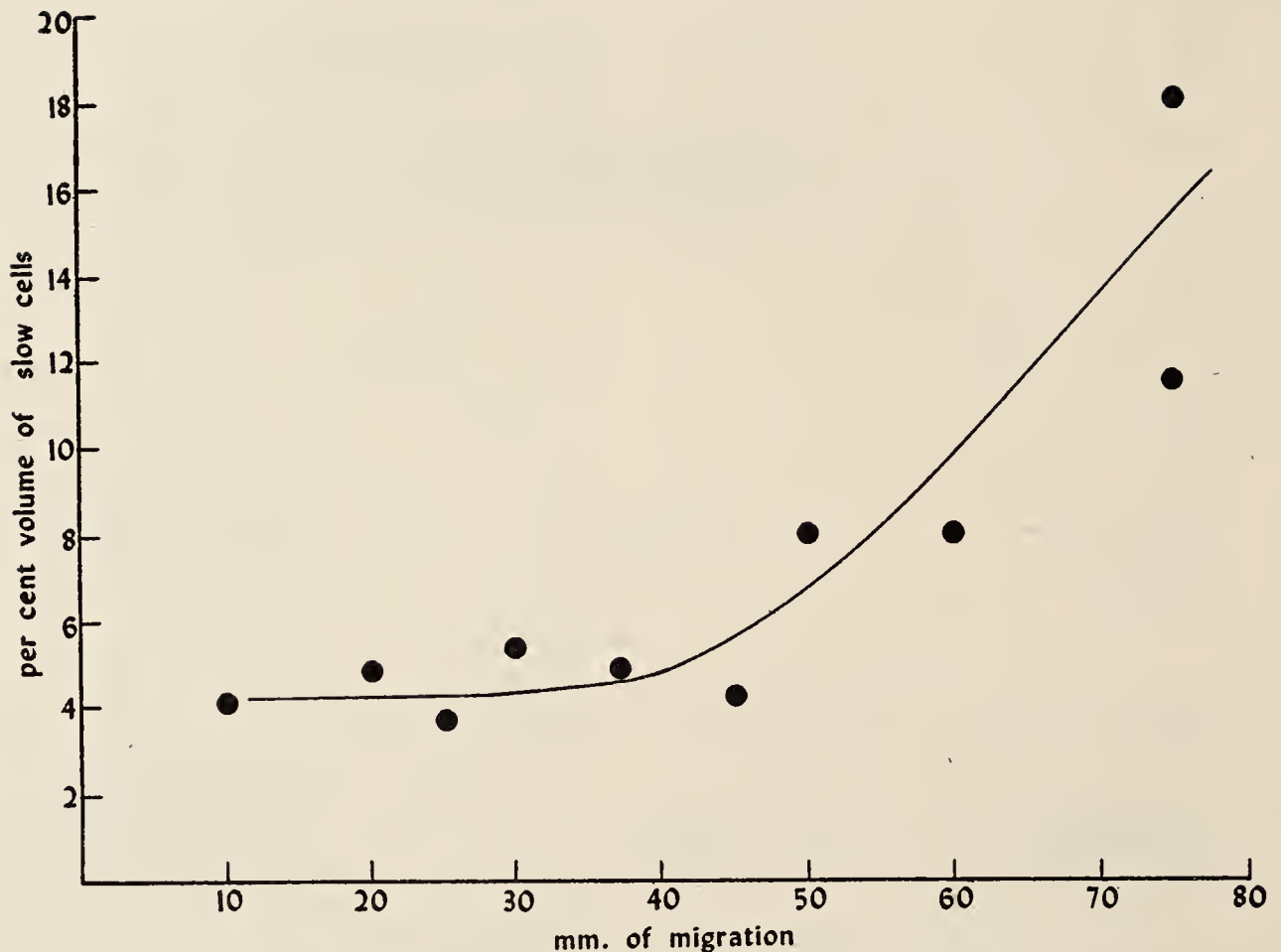


FIG. 7. THE PROGRESSIVE ACCUMULATION OF SLOW CELLS IN THE POSTERIOR END OF *D. MUCOROIDES* AFTER PROLONGED MIGRATION

Each point represents a mean of about 10 pseudoplasmodia.

In this case  $y$  and  $x$  are the pre-spore and pre-stalk volumes respectively,  $k$  is the slope of the line, and  $b$  is a constant. In glancing at Fig. 3, it may be seen that in *D. discoideum* the slope is approximately 1, while in *D. mucoroides* the slope is greater than 1. In the strain DM-4 the slope appears to be similar to other strains of *D. mucoroides*, but differs in the constant  $b$ .

With the generous help of Dr. Martin Wilk of the Mathematics Department of Princeton University, it has been possible to check these assertions statistically. It was found desirable, in this analysis, to plot the logarithm of the pre-spore volume against the sum of the log of the pre-spore and the log of the pre-stalk volumes, for in this way the variability is confined mainly to one variable, namely, the pre-spore volume (Fig. 9). On such a plot the slope of the *D. discoideum* curve should now be 0.5, and the slopes of the *D. mucoroides* curves should be  $>0.5$ . Using a least square analysis, the slope of *D. discoideum* is  $0.4865 \pm$

$0.0488$ , *D. mucoroides*  $0.5361 \pm 0.0126$  and DM-4  $0.5295 \pm 0.0180$ , where the allowance is twice the standard error of the estimate.

By means of significance tests (see Appendix) it is possible to say that we may reasonably assume that the slope of *D. discoideum* is 1 (when plotted allometrically as in Fig. 3), and also that each of the two *D. mucoroides* strains has the same slope and that this slope is greater than 1.

Now comes the problem of extending the basic hypothesis concerning proportions so that these new facts may be included. Let us assume that the polar factor ( $S$ ) can be used in two related reactions, one involving stalk formation and the other involving the formation of the characteristic pre-stalk condition that results in the formation of a division line (Fig. 10). There are here three processes whose rates will be important in the over-all mechanism: the rate of polar movement and therefore the supply of  $S$  ( $k_1$ ), the rate of utilization of  $S$  in stalk formation ( $k_2$ ) and the

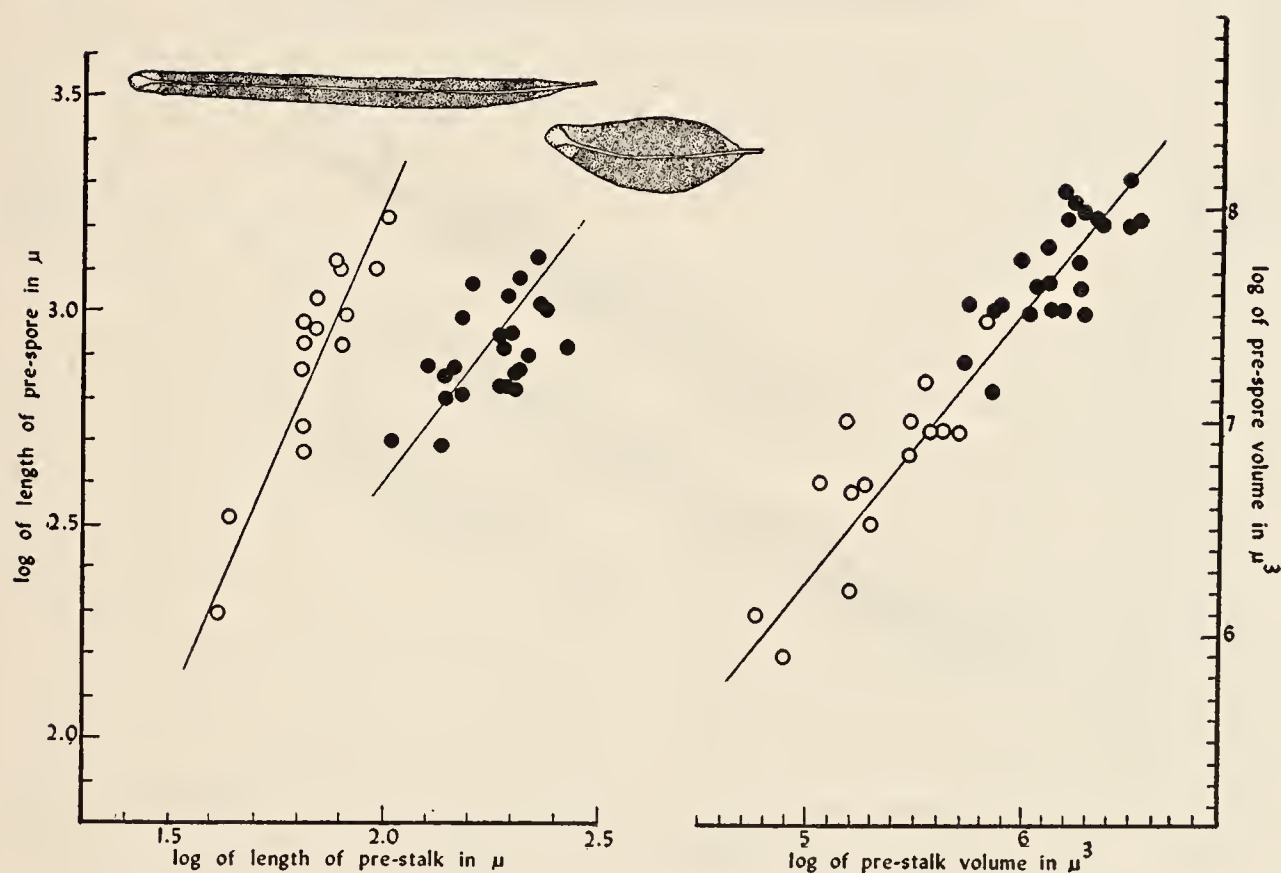


FIG. 8. A COMPARISON OF THE LOGARITHMIC PLOTS OF LINEAR MEASUREMENTS OF THE PRE-SPORE AND PRE-STALK ZONES (LEFT) WITH THE VOLUMES OF THE SAME ZONES (RIGHT), FOR BOTH THIN AND FAT CELL MASSES OF *D. MUCOROIDES*

Note that when the volumes are used the data fall into one continuous curve.

rate of utilization of  $S$  in pre-stalk formation ( $k_3$ ). We must also make the assumption that the volume of the pre-spore mass ( $y$ ) varies directly with its ability to deliver  $S$  into the anterior region ( $k_1$ ), and that the volume of the pre-stalk mass ( $x$ ) varies directly with the utilization of  $S$  in the pre-stalk reaction ( $k_3$ ). That is,

$$y \propto k_1$$

$$x \propto k_3$$

Now in the case of *D. discoideum* there is no stalk formed during migration. Therefore the amount of pre-stalk is completely dependent upon the rate of supply of  $S$ . That is,

$$k_1 = k_3$$

$$\therefore \frac{y}{x} \propto \frac{k_1}{k_3}$$

$$\text{or } y \propto \frac{k_1}{k_3} x$$

In other words, the relation of  $x$  and  $y$  for *D.*

*discoideum* is linear, giving a slope of 1 when  $x$  and  $y$  are plotted logarithmically. This is consistent with the observed facts. (There is no reason to plot the data logarithmically except for the purpose of comparing it to *D. mucoroides*).

In the case of *D. mucoroides*, which continuously forms a stalk during migration, we assume that the polar factor  $S$  is channeled in two directions in the formation of both stalk ( $k_2$ ) and pre-stalk ( $k_3$ ). There is therefore less  $S$  available for pre-stalk and, accordingly, the pre-stalk of *D. mucoroides* is smaller (as shown in Fig. 2). The relation between pre-spore and pre-stalk volume is in fact an allometric one (as shown in Fig. 3).

Since the slope of the line when plotted logarithmically is greater than 1, we must make a further assumption concerning the relation of the polar factor  $S$  to the volumes of  $x$  and  $y$ . There are numerous possibilities; for instance, we could say that the process of stalk formation in some way causes a nonlinear delivery of  $S$  into the pre-stalk zone. That is, the amount of  $S$  available for pre-stalk material does not have a linear, but

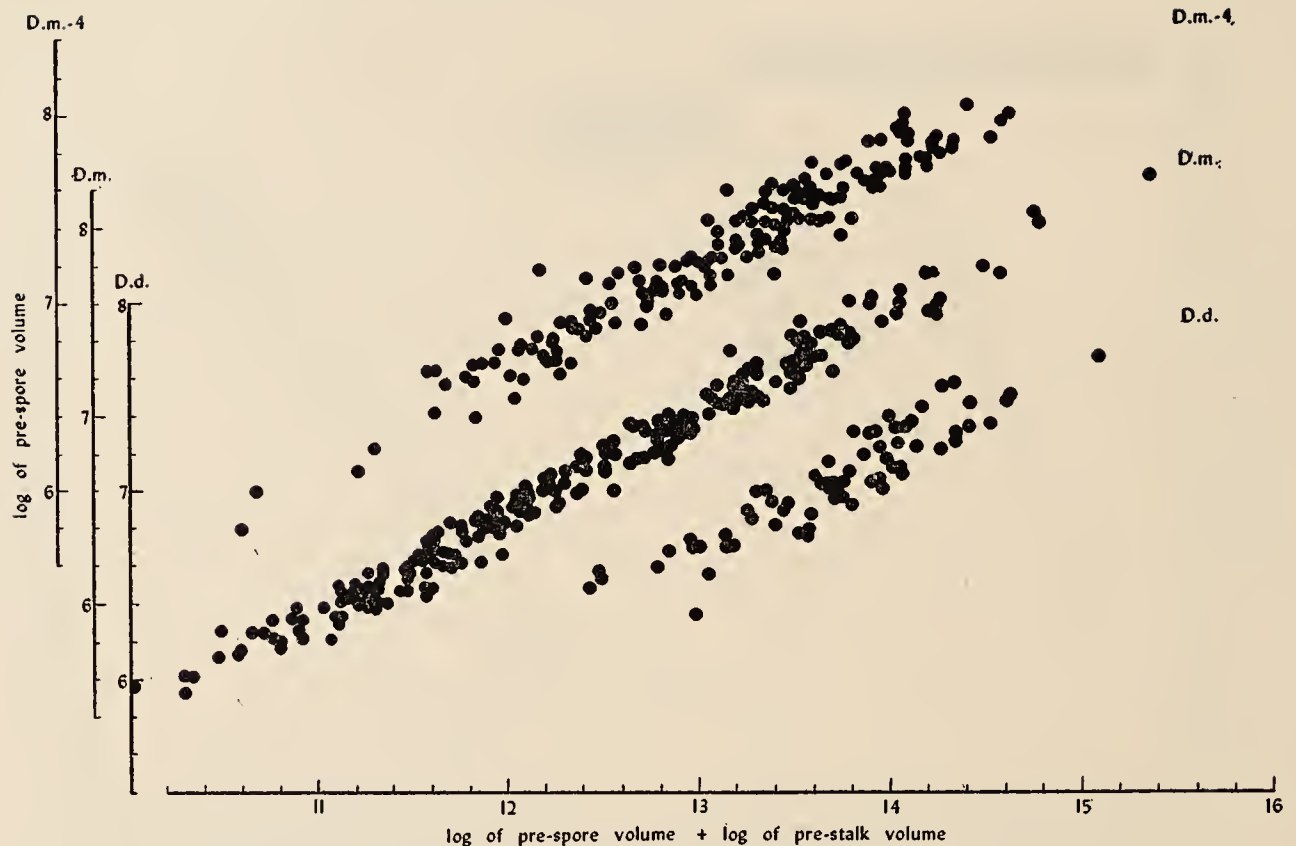


FIG. 9. A LOGARITHMIC PLOT OF THE PRE-SPORE VOLUME AGAINST THE SUM OF THE PRE-SPORE VOLUME AND THE PRE-STALK VOLUME

Reading down the curves are for DM-4, other *D. mucoroides*, and *D. discoideum*.

rather an exponential, relation to the size of the cell mass. But since the details of the mechanism are unknown, it is perhaps only useful at the moment to remember that in *D. mucoroides*, where the stalk is present, there is a reduction in the size of the pre-stalk volume, and that this can easily be interpreted in terms of the proposed polar factor scheme.

In comparing the two strains of *D. mucoroides*, it may be assumed that for some reason in DM-4 the percentage of fast cells is low relative to the total mass. This means that if two cell masses, one of DM-4 and the other of DM-11, are to deliver an equivalent amount of *S* in the anterior portion, then their anterior portions (*x*) will be equivalent in size because in both cases the utilization of *S* will be equal ( $k_3$ ). From one previously mentioned assumption ( $x \propto k_3$ ) this would result in equal pre-stalk zones (*x*).

However, we have suggested that in DM-4 the number of cells required to produce this equivalent amount of polar factor (*S*) is comparatively large. Therefore the volume of the pre-spore areas (*y*) would not be equal, but greater in DM-4. Since the kinetics of the reactions are in no way

affected, one would not expect a change in the slope of the line, but there would be a change in the constant *b*, which conforms with the experimental results. (It is interesting to note here that the DM-4 strain is somewhat special in its periodic acid-Schiff staining properties. It shows a gradient of staining, with the greatest intensity at the anterior end (as described for *D. mucoroides* in Bonner, Chiquoine, and Kolderie (1955), while the other strains showed a more uniform staining, with an abrupt zone of light cells at the posterior end (see Fig. 2.)

The only case not mentioned is that of *Polysphondylium*. Here, because of some alteration in the timing, some delayed maturity, there is no pre-stalk zone: Therefore all the *S* is used directly in stalk formation, and there is no division line to measure.

In summary, the theory presented here is that there is a polar factor which is involved directly in the formation of the pre-stalk condition as well as the stalk itself, and that the different proportionality characteristics of different species and strains can be directly accounted for by such an hypothesis. It may now be helpful to consider



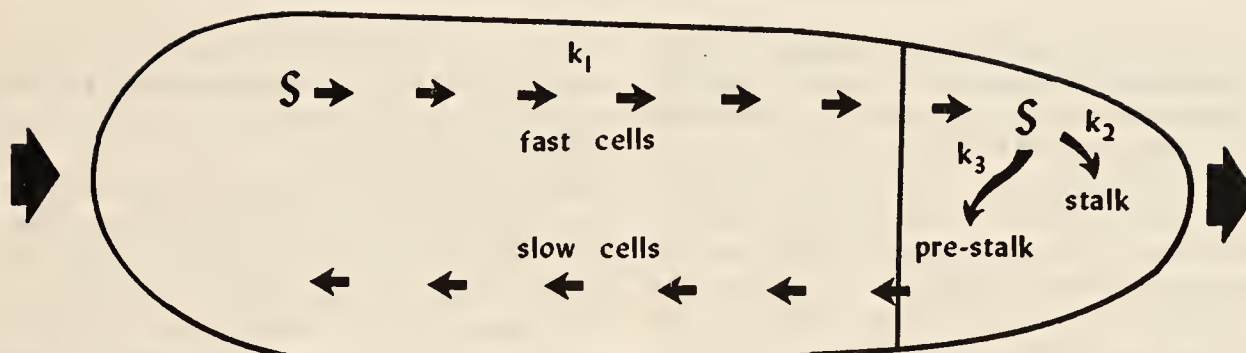


FIG. 10. DIAGRAM ILLUSTRATING THE HYPOTHESIS OF POLAR CONTROL OF DIFFERENTIATION IN SLIME MOLDS

$S$  indicates a polar factor which moves forward at rate  $k_1$  and is used to make stalk at rate  $k_2$  and pre-stalk at rate  $k_3$ .

this theory in the light of the evolution of the cellular slime molds.

#### THE EVOLUTION OF CELLULAR SLIME MOLDS

The first step in considering the evolution of slime molds is to speculate on their adaptive value over free-living amoebae. This step is in many ways the most difficult because we are forced to make the gratuitous assumption that they are adaptively superior; yet there is little or no evidence to show that this is the case. Free living amoebae which are incapable of forming cysts, others which are, and the communal slime molds all live side by side in the soil and must have done so for millions of years.

There are some conceivable advantages to the aggregation behavior of slime molds. For one thing, it may be that by coming together in groups in the very beginning certain deficiencies were masked, and as with syntropism in bacteria, or heterocaryosis in fungi, the pooled resources of a number of cells may have resulted in a sort of mutual dependence. Another point is that, in all cases of cellular slime molds, morphogenesis spatially separates the feeding, vegetative stage from the fruiting, sporulation stage. Feeding, which involves the phagocytosis of bacteria by thin, delicate amoebae, must occur in an aqueous medium. The formation of spores, on the other hand, involves a desiccation of the amoebae, which become encapsulated, and this conceivably could be more efficiently performed in the atmosphere (although many free-living amoebae form cysts in water). More important is the idea that by lifting the mass away from the region of feeding, a process which is abetted by the light and heat tropisms, the spores are in a more favorable position for dispersal. The cellular slime molds

would certainly seem to have a more effective method of spreading than free-living amoebae.

With these basic assumptions as a foundation, let us now trace a hypothetical sequence of events from the separate, isolated amoebae to the more complex forms. The first step may have been a sensitivity to certain substances given off by their own kind, which led ultimately to the acrasin-controlled aggregation. Aggregation involves a polar movement of cells and with this polarity comes a gradient: a high activity in the front end of the cell mass which decreases posteriorly.

The next step might have been the production of a pedestal to isolate the spores from the substratum. Relatively solitary amoebae such as *Sappinia* may have individual stalked cells or cysts, but more interesting is the recent discovery by Raper (1956) of the new genus *Acytostelium*. This form has a simple, although not highly organized, aggregation, and the resulting small cell mass rises into the air and exudes an extremely delicate cellulose cylinder that is devoid of any stalk cells. The point is that all the cells produce stalk material and then subsequently they all produce spores; there is no division of labor.

If now, in an *Acytostelium*-like ancestor, there was an increase in variability within the cell mass (and the importance of this supposition cannot be overemphasized), then one would expect the beginning of a sorting out of fast, perhaps high-energy, cells and slow, low-energy cells. With this differential ability to move would come a differential ability to produce or contribute in the stalk or in the spore direction.

One has to imagine that there has been some sort of facilitation in this sorting out, that is, the anterior reaction in particular has become exaggerated, perhaps simply because fuel is now constantly poured on the fire. But the process is so

energetic that the fast cells become virtually consumed; they become incapable of further propagation and contribute their hollow frames to give further support to the stalk they have manufactured. At first there is a continuously variable population of fast and slow cells, but by this facilitation connected with the anterior reaction, the fast cells produce a totally new cell type: the pre-stalk and the stalk cell. Therefore, the continuous variation results in a discontinuous one, for the labor is finally divided into the two functions: spore formation and the morphogenetic activity of stalk formation.

The situation described thus far applies to *D. mucoroides*. *D. discoideum* has made one further, obvious step forward. For organisms that have ceased all feeding before aggregation, cellulose, which surrounds the stalk, is a precious substance. To migrate 100 mm. or more is a great expense in both cells and cellulose for *D. mucoroides*, while *D. discoideum* can cover the same distance without forming any stalk. Therefore, by changing the timing of the various steps that lead to maturity, it has been possible to migrate greater distances more economically.

In many ways *Polysphondylium* presents the greatest enigma. Again, by an alteration of timing, that is, by delaying the preliminary steps toward maturity until the very last moment, this form has been able to produce a fruiting body with many side branches. But here the adaptive advantage seems especially hard to grasp. Perhaps by breaking up the number of sori into small branches the effectiveness of dispersal is increased, but this is an uncertain hypothesis at best.

In conclusion, it might be said that in these proposed evolutionary steps the most interesting advance is the idea that the cell variability within an organism may increase, but always necessarily in a continuous fashion. Then, since the variation involves a polar activity (i.e., polar movement), there is an opportunity for reactions to exist at one end of the cell mass that are absent in the other. Therefore, the continuous variation turns into a discontinuous one, and the result is a division of labor, a differentiation. Furthermore, the fact that there is a balance between the rates of polar movement and the rates of the anterior reaction means that the spacing of differentiation is controlled and proportionality is the result.

#### SUMMARY

A basic problem in the study of the development of slime molds is an explanation of their propor-

tional development. It has been possible to work out a theory which satisfactorily accounts for the many experimental observations that have been gathered on this subject.

Differentiation begins in the sausage-shaped cell mass, and from previous work it is known that there are certain specific chemical differences between the anterior, pre-stalk end and the posterior, pre-spore end. Furthermore, from new observations presented here, it is now known that these presumptive areas are proportionate in cell masses of different size and even in parts of a cell mass that has been cut into fractions.

Clearly, then, a cell in the anterior end "knows" how many cells lie posteriorly, for if they are removed experimentally the anterior cells accommodate proportionately to the new loss. The theory assumes that this information is carried by a polar movement; in fact, it specifically suggests that certain fast cells are constantly arriving at the tip, and if there is a reduction of the posterior portion then there is a corresponding reduction of these messenger cells.

The "message" carried may be simply a supply of food necessary for a reaction taking place at the anterior end. This anterior reaction is the one which is responsible for the formation of the pre-stalk and stalk condition. In any one species, then, there is a balance between (1) the rate of delivery of the polar factor to the anterior reaction and the rate at which this reaction produces (2) the stalk and (3) pre-stalk portions. Different species and strains have different proportionality characteristics, and these differences could be accounted for by differences in the rates of the three above-mentioned processes.

In considering the evolution of such a mechanism of differentiation, the first step might have been an increase of variability of the cells within the cell mass with respect to their stalk-forming and spore-forming abilities. Since the cell movement in such masses is polar (presumably because of the acrasin mechanism and pull tensions among the amoebae), the fast and slow cells sort out, and such concentration of one cell type has led to a facilitation or an amplification that is the anterior reaction. Therefore, random variability in a cell mass has ultimately, according to this hypothesis, produced a discontinuous, proportionate cell difference that has all the qualities of a controlled pattern of differentiation.



## APPENDIX

1. *Modification (for slime molds) of V. R. Gregg's Methylene Blue Technique.* The pseudoplasmodia are fixed for 1 to 2 hours in a solution of 5 ml. of formalin in 95 ml. of 70% ethyl alcohol. The staining is for an hour in 0.25 g. of methylene blue, 100 ml. of 70% ethyl alcohol, and 0.5 ml. of conc. HCl. This is followed by a very short interval in absolute alcohol and then in methyl salicylate, where they remain for 3 to 24 hours. Before mounting in clarite, they are put through a short change in fresh methyl salicylate followed by 5 minutes in toluene.

2. *Summary of the results of the significance tests of the slopes of the lines indicated in Fig. 9.* A formal test of significance of the hypothesis that the slope of the *D. discoideum* curve is 0.5 gives a *t* value of 0.553. This is obtained by taking the difference between the observed and the theoretical slopes, and dividing this difference by the standard error. Since 0.553 lies between the 80 and the 50 per cent points of the *t* distribution for 61 degrees of freedom, it is clear that there is no reason to invalidate the theoretical value of 0.5.

In the same way a test of the significance of the hypothesis that the slopes for DM-4 and the other strains of *D. mucoroides* are each 0.5 is

again obtained by calculating the values of *t*, which are found to be 3.21 and 5.89 respectively. Since 3.21 (and hence 5.89) lies beyond the 0.2 per cent point on the *t* distribution for 180 and 249 degrees of freedom, the data are clearly inconsistent with the hypothesis tested. Therefore, we can conclude that the slopes of the lines for all the *D. mucoroides* strains tested are larger than 0.5.

Finally, it is possible to test the hypothesis that the slopes of DM-4 and the other strains of *D. mucoroides* are the same. The value for *t* in this case is 0.5689, which lies between the 80 and the 50 per cent points of the *t* distribution for 180 and 249 degrees of freedom. Clearly there is no basis to doubt the hypothesis that the two slopes are the same.

## ACKNOWLEDGMENTS

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## Cell Mixtures of Different Species and Strains of Cellular Slime Moulds

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*From the Department of Biology, Princeton University*

WITH ONE PLATE

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### INTRODUCTION

THE cellular slime moulds have, in their life history, a period of feeding of separate independent amoebae which subsequently stream together (by a process involving chemotaxis) to central collection points. Each of the resulting cell-masses differentiates into a fruiting body; in most species there is a delicate tapering cellulose stalk which surrounds the large vacuolate stalk-cells, and an apical sorus containing a considerable quantity of individually encapsulated spore-cells.

There are a number of distinct and easily recognizable species and, moreover, it is a common observation (see Raper, 1951) that different isolates from the soil of any one species may have small but identifiable characteristics. It was found, for instance, in a previous study (Bonner & Shaw, 1957) that some strains under given environmental conditions formed a very long stalk, that is, delayed the process of spore differentiation, while others show rapid spore differentiation and correspondingly short stalks.

The purpose of this study was to examine the effect on the morphogenesis of these slime moulds of mixing the cells of different species and different strains in various ways. Such experiments show that there are varying degrees of compatibility among the combinations and that in many cases there are interesting types of compound fruiting bodies. Also it will be demonstrated that cells of a particular strain when disassociated tend to regroup within the cell-mass and certain parallels will be drawn between these results and those of sponge and other animal cell-dissociation experiments.

### MATERIALS AND METHODS

The organisms were grown at 22° C. on plain, non-nutrient 2 per cent. agar upon which a loopful of *Escherichia coli* had been spread thin, a modification of a technique of Singh (1946) previously suggested to us by B. M. Shaffer. The experiments were run at room temperature (25° ± 3° C.).

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The following species and strains were used:

*Dictyostelium discoideum*. This species is characterized by a stalkless migration period at the end of which stalk formation begins. The resulting fruiting body has a short stalk, a circular basal disk at the base of the stalk, and a white sorus. Only one strain of this species was used (Dd-1).

*D. mucoroides*. This species begins stalk formation at the end of aggregation and continues to build stalk throughout its migration period. Of the four strains used, Dm-2 invariably has a short migration phase, while Dm-4, Dm-11, and Dm-16 all have long migration under the conditions used. The latter three strains have minor morphological differences (e.g. Dm-4 has a serpentine rather than a straight stalk during migration) but these differences are immaterial to the present study.

*D. lacteum*. Similar to *D. mucoroides* except that it is very small, has virtually no migration, and its spores are round instead of capsule-shaped. Only one strain was used (Dl-1 from K. B. Raper).

*D. purpureum*. The same as *D. mucoroides* except that the final sorus is purple in colour. Only one strain was used (Dp-2) which possesses the character of long migration.

*Polysphondylium pallidum*. During the beginning of its migration this species resembles *D. mucoroides*, although eventually it pinches off a series of cell-masses on the stalk, each one of which produces a group of small fruiting bodies coming off the main stalk as branches in a whorl. There is also one large terminal sorus which is white, as are all the secondary sori. Only one strain was used (Pp-2).

*P. violaceum*. The same as *P. pallidum* except that the sori are purple. Two strains were used, of which Pv-1 has slightly shorter migration than Pv-2.

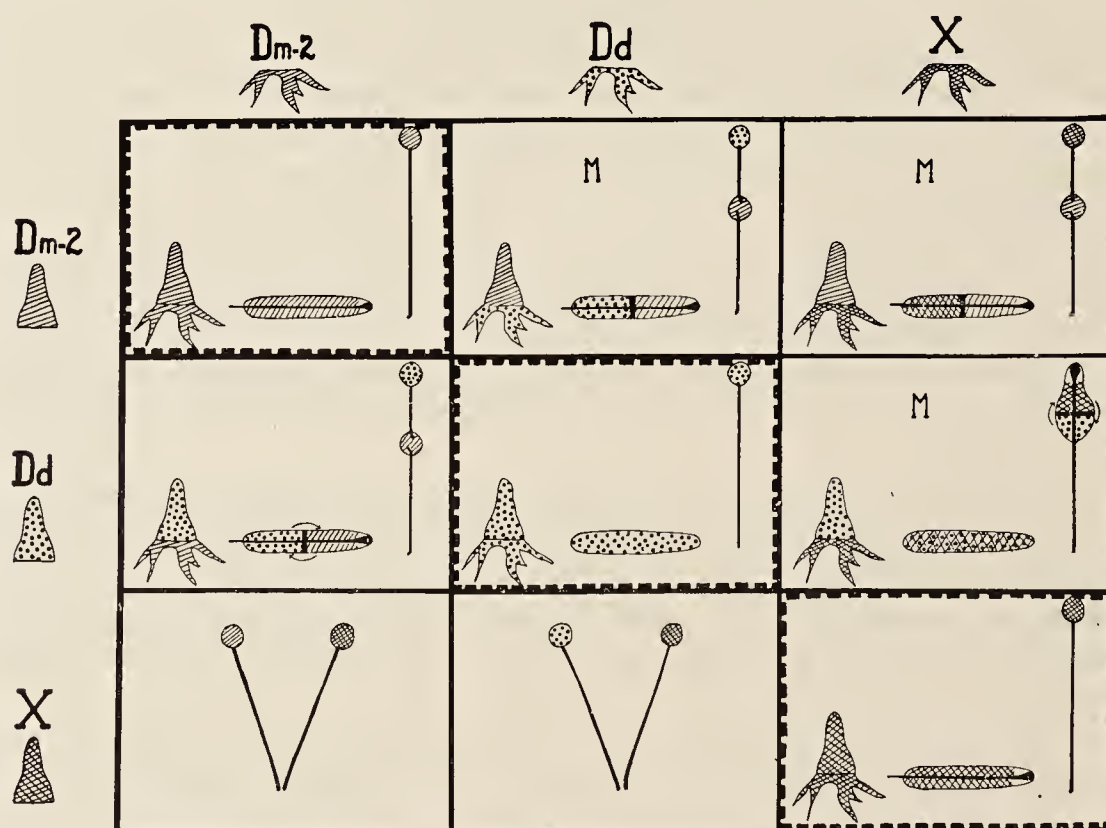
## RESULTS

### *Grafts in the aggregation stage*

The cells of these species and strains were mixed in three different ways. In the majority of the experiments the following procedure was used. First the centre of an aggregation pattern of one strain was removed with a hair loop. Then the centre of an aggregation pattern of another strain was placed in the middle of this aggregate. If there was any degree of compatibility at all the aggregating streams would join the foreign cell-mass, so that presumably approximately the anterior half was one strain and the posterior half another.

For the sake of simplicity of presentation it may be useful to the reader to follow this discussion by referring to Text-fig. 1. As indicated in the margins of this diagram, there are three groups of strains that have discrete reactions: (1) Dm-2; (2) Dd-1; (3) Dm-4, Dm-11, Dm-16, Dp-2, all of which for convenience are designated X. If, now, a Dm-2 anterior end is grafted on to another Dm-2 posterior aggregation stream, then the result (Text-fig. 1, upper left box), will be a normal complete Dm-2 fruiting body. Similarly, Dd grafted to Dd will give

a normal Dd (central box), and an *X* grafted to an *X* will give a normal *X* (lower right-hand box). In this latter case this is only true if the *X* is the same strain in both anterior and posterior portions (e.g. Dm-16 grafted on to Dm-16), for any other combinations of *X* (e.g. Dm-16 grafted on to Dm-11) invariably result in a complete separation of the cells to form two distinct fruiting bodies, showing no tendency to merge. This point will be touched upon again later.



TEXT-FIG. 1. A diagram illustrating the results of the grafts made during the aggregation stage. The centre of one strain (indicated on the left) is placed on the decapitated aggregation pattern of another strain (indicated on the top). If the graft involves the same strain from both parts the result is normal development (indicated by the three squares with serrated borders). *M* designates those combinations which were also tested by mixing the cells at the migration stage. For further details see the text.

*Dm-2 in front of Dd.* If the anterior end of Dm-2, which is a short migration form, is grafted on to the posterior portion of Dd, a form which possesses the power of prolonged migration, then the two cell-masses will stay fused, but the final result, in all 8 cases tried, is a double fruiting body (Text-fig. 1, centre top box). It could readily be observed that the first fruiting body to form was the anterior Dm-2 (which exhibits short migration and rapid spore differentiation), and that the previously posterior Dd pushes past the final spores of the Dm-2 and either forms a fruiting body directly on top of the sorus of the Dm-2 (as shown in the diagram), or migrates away on to the agar substratum and fruits



elsewhere. The fruiting bodies of the two species are sufficiently distinct, so that following the separate strains from the merged mass is relatively easy. More detailed studies, including the use of paraffin sections, were made on the next combination, the results of which are essentially similar.

*Dm-2 in front of X.* This combination (Text-fig. 1, upper right box) again uses the Dm-2 tips, but in this case the aggregating patterns of the various members of the *X* complex were placed in the posterior ends. The reason, as stated above, for the use of this *X* grouping is that all members of the group behave the same way (31 cases with Dm-4, 4 with Dm-11, 6 with Dm-16, 4 with Dp-2).

The fact that the anterior, rapidly differentiating Dm-2 formed a fruiting body first, and the *X* pushed through to form the 'second story' fruiting body, was seen especially strikingly when Dp-2 was used for the *X*. Since this species has a purple sorus, the lower sorus was pure white and the upper sorus purple, clearly indicating that the posterior Dp-2 had passed through the Dm-2 cell-mass. This was further tested by taking the spores from the upper and lower sori, culturing them, and examining the progeny, and there was no evidence of intermingling of cell-types.

The mechanism of this 'passing through' was then examined by the use of paraffin sections. As was known from a previous study, the periodic acid-Schiff technique is especially useful at revealing the state of differentiation of a migrating mass. (For the details of the method see Bonner, Chiquoine, & Kolderie, 1955.) Sections were prepared of the Dm-2 to *X* grafts at various stages and, as shown in the Plate, fig. A<sub>1</sub>, there is a sharp, discrete division line between the two strains. The *X* strain shows no differentiation whatsoever until the Dm-2 undergoes its final differentiation. Then, as shown in the Plate, fig. A<sub>2</sub>, the *X* shows pre-spore and pre-stalk zones as well as the beginning of stalk formation at the tip. It quickly pushes past the differentiated spores of the Dm-2, using the sorus of Dm-2 as a platform. The stalks of the two strains never join; the tip of the Dm-2 stalk will lie near the base of the *X* stalk.

*Dd in front of Dm-2.* Another combination possible is to put Dd tips in the centre of Dm-2 aggregation streams (Text-fig. 1, middle, left box), and this, it will be noted, is the reverse of the first experiment. Here the rapidly differentiating Dm-2 is initially behind the long migration Dd, yet the end result is the same as before, namely, the Dm-2 produces its fruiting body on the agar, and the Dd again has the upper, 'second story' position. (This was true in 15 cases, in 11 others the separation was complete from the beginning.) In a number of the cases the spores were removed from the two sori and their progeny tested, which completely confirmed the apparent composition of the two fruiting bodies.

In some of the cases, for an interval following aggregation, there was a stalkless migration which is characteristic of the anteriorly placed Dd. In other cases stalk formation occurred at the very beginning and gave the general appearance of Dm-2. This puzzling behaviour was again analysed by making stained

sections, and it was found that fairly rapidly following the end of aggregation there is a redistribution of the strains; in some way the Dm-2 cells assume a more anterior position. There is a suggestion from the slides (Plate, fig. B) that this occurred by small groups of Dm-2 cells passing through the Dd cells. There is no doubt, however, that the Dm-2 cells did become anterior, with the result that they formed the first lower fruiting body, and the Dd climbed past them to build the upper fruiting body.

*Dd in front of X.* Placing Dd centres into the aggregation streams of *X* gave a more complete combining of the two strains, when the experiment was successful. In 35 cases, 14 showed complete separation and the remaining 21 gave a single fruiting body. If spores were tested from the single sorus of one of these combined fruiting bodies they gave both parental types. Occasionally the spores of one of the two would differentiate sooner and be left behind on the stalk, thus superficially giving the appearance of a double fruiting body, though in fact this was only a single stalk with a ring of spores about its middle.

In the stained sections of this combination there was no sharp division line between the two strains during the migration period; the cells appeared to intermingle. The migration was characteristic of Dd, showing no stalk formation. As the final spore differentiation approached it was obvious, however, that the pre-spores of each strain had sorted out into two cohesive, compact cell-masses, one lying adjacent to the other (Plate, fig. C). There was no consistency as to the sequence, for in some cases the Dd pre-spore mass was anterior, and in others it was the *X* pre-spore mass. There is no way at present of ascertaining whether one or both of the strains contributed cells in the formation of the stalk.

*Dd in front of Dd.* Some of the above experiments were done with Dd stained with vital Nile blue sulphate (Bonner, 1952), and it was noticed that when a blue tip was grafted on to a white aggregation stream of another strain there was a rapid mixing of the blue, so that the whole sausage-shaped cell-mass was blue by the beginning of migration. To see whether this rapid mixing normally occurred within any one strain, the same experiment was repeated by grafting blue tips of Dd on to colourless aggregation streams of Dd and vice versa. Invariably the resulting cell-mass would be uniformly blue, indicating a violent mixing of cells at the end of aggregation. This result is surprising, since it is well known that when the graft is made later, during the migration stage, the division line remains fairly constant for a number of hours (Raper, 1940, 1941; Bonner, 1952, 1957). Apparently the end of aggregation is a critical period of cell distribution and should be investigated further for its role in morphogenesis.

*All other combinations.* A number of other combinations were tried, and in every case there was a complete separation of the two strains to form two fruiting bodies. In Text-fig. 1, *X* grafted on to Dm-2 (23 cases) and *X* grafted on to Dd (18 cases) gave such a result. As mentioned before, all the members of the *X* complex (Dm-4, Dm-11, Dm-16, Dp-2) were tried against each other in reciprocal crosses with no mergers. Also *Dictyostelium lacteum* was combined with Dd,



Dm-2, Dm-4, and *Polysphondylium violaceum* (Pv-2). Finally, Pv-1, Pv-2, and *P. pallidum* (Pp-2) were combined in various ways with the same lack of unification of the cells.

#### *Cell mixtures at the migration stage*

The second of the three methods used to combine the strains was to take two young migrating masses, each from a different strain, lop off the anterior  $\frac{1}{10}$  of each, and thoroughly mix the two with an eyelash attached to a glass tube so that there was one small compact circular blob of mixed cells. The mixtures made are indicated by the letter *M* in Text-fig. 1.

*Dm-2 and Dd mixtures.* Mixing appeared to give an even closer association than did grafting, for in 7 cases out of 10 there arose a single fruiting body which contained the spores of both strains (the remaining 3 cases were too confused to interpret). Fig. D of the Plate shows a stained section of such a combined fruiting body, and it is obvious that the cells of each strain have regrouped into discrete, though cohesive, masses of pre-spores. There was no stalkless migration characteristic of Dd.

*Dm-2 and X mixtures.* With this combination the result was identical with the grafting experiments, there was a sorting out to the extent that a double fruiting body was produced in which the lower sorus was Dm-2 and the upper sorus was *X* (which was demonstrated by replating of the spores (11 cases)).

*Dd and X mixtures.* As with the grafts, a single fruiting body was produced in which the cells of each strain regrouped themselves within one cohesive mass of spore-cells (10 cases). It should be noted here that this experiment is identical with that of Raper & Thom (1941), who mixed migrating masses of *D. discoideum* and *D. purpureum*, obtaining fruiting bodies made up of the cells of both species.

*X and X mixtures.* With one exception, no other mixture fused in any permanent way and separate fruiting bodies would result. In one case out of 7 (Dm-16, Dp-2 mixtures) there was a clearcut double fruiting body in which Dm-16 formed the lower one, and Dp-2 the upper one.

#### *Grafts at the migration stage*

A few grafts were made at the migration stage by grafting the anterior half of the migrating mass of one strain on to the posterior half of another. This was tried with Dm-2 grafted on to Dd (2 cases) and *X* (4 cases) and, as with the aggregation grafts, they gave rise to double fruiting bodies. The only point of interest here is that when Dd was placed in front of Dm-2 they separated completely (3 cases). It will be remembered that with the aggregation grafts (Text-fig. 1, left, middle box) the Dm-2 crawled ahead of the Dd to assume an anterior position, and that from the vital blue Dd's grafted on to colourless aggregation streams of Dd there was evidence of violent cell redistribution at the end of aggregation. Therefore this separation of Dm-2 and Dd at the migration stage is



consistent, for the grafting has been made after the period of active cell redistribution, and there is no longer any opportunity for the Dm-2 cells to surge forward and intermingle with the cells of the Dd.

#### DISCUSSION

The mixing of cells of different species, particularly at the feeding or vegetative stage, was first performed many years ago by Olive (1902). He says: '... two well marked species of *Dictyostelium*, one for example with white spores and another with dark, sown in the same spot of a nutrient agar tube, will result in fructification showing the two distinct forms growing side by side without any trace of intermixture.' This matter was thoroughly investigated by Raper & Thom (1941), who not only confirmed Olive but greatly extended the observations. They showed that mixed spores or vegetative amoebae of *D. discoideum* and *P. violaceum* gave separate aggregations, while mixtures of *D. discoideum* and *D. mucoroides* formed common ones, but separate fruiting bodies were formed at the centre. Grafts during the migration stage displayed some temporary coalescence in the combination of *D. discoideum* and *D. purpureum*. Furthermore, they found that these same two, as mentioned above, would form a unified fruiting body when their migrating cell-masses were thoroughly intermixed.

Gregg (1956) has pursued this matter of adhesion between cells by injecting amoebae of different species into rabbits and obtaining 'anti-amoeba' antibodies. He found that the antibodies were species specific with vegetative amoebae, but that this was not the case once the aggregation stage was reached, and on the basis of these experiments he suggests that a general tendency for surface adhesion appears at the aggregation stage, thereby possibly explaining the observation of Raper & Thom (1941) that the combining of *D. discoideum* and *D. purpureum* cells can only take place at later stages of development. It is, of course, difficult to know to what extent Gregg's experiments are revealing properties of the cell surface, although there is no doubt, as Shaffer (1957 *a, b*) has shown in a series of most interesting observations, that the stickiness of cells, along with specific differences in the acrasin (or chemotactic substance) of different species, plays an important role in the aggregation and cell association phases. Furthermore, he has shown that certain combinations of species adhere more readily to one another than others.

One of the curious facts that comes from our experiments is that specific differences between strains of one species are as great, or even greater, than those between different species. Certain strains of *D. mucoroides* were completely unable to permanently coalesce with one another, and this was true as well of the two strains of *P. violaceum*. Yet partial merging was possible between *D. discoideum* and particular strains of *D. mucoroides* and *D. purpureum*. Each strain represents a separate isolate from nature, and M. F. Filosa from our

laboratory is now in the process of further analysing the nature of these strains and their genetic constitution.

The results obtained upon mixing strains are obviously not merely a matter of surface specificities. This is particularly evident in the case of Dm-2, where in part its unique properties may be considered in terms of its rate of differentiation. The fact that in this strain spore differentiation occurs so quickly means that it will supply, in a short time, a base for the second fruiting body. In other words, the basis of the double fruiting body formation is the differential between the rate of spore formation in Dm-2 and its partner (Dd or all the strains that comprise X). It is especially interesting that the rate of differentiation of two groups of cells lying side by side (e.g. Plate, fig. A) can be so completely independent; it indicates that this rate is intrinsically controlled within the cells.

Another noteworthy point is that when Dm-2 leads the cell-mass (Plate, fig. A<sub>1</sub>) the posterior strain remains totally undifferentiated for an abnormally long period; histologically it appears to be in an aggregating condition despite the fact that it may have been part of a migrating or culminating mass for some hours. It only regains its ability to differentiate after the Dm-2 has completely sporulated. This is clear evidence that the anterior Dm-2 holds back the development of the other strain.

If we now examine the interesting property of the sorting out of cells within a cell mixture, it is possible to classify the results into three degrees of the extent to which the cells of two strains pull away from one another—three degrees of cell compatibility. (1) At one extreme the cells separate completely to form two separate fruiting bodies. (2) In the intermediate case double fruiting bodies are formed, which is characteristic when Dm-2 is present. (3) The highest degree of compatibility found thus far is when a single sorus contains two separate, cohesive patches of spores, each belonging to one of the strains. Since the cells in this case do still pull apart and regroup there is obviously some incompatibility, and theoretically it should be possible to find two strains that are completely compatible in which the spores of both will spread at random through one common sorus.

This matter of sorting out is now a well-recognized phenomenon in experiments with dissociated animal cells. This is no place for a detailed history of the discovery of the phenomenon, but the original idea stems from the work of H. V. Wilson (1907), who pushed sponges through bolting cloth and noted that the dissociated cells reorganized to form a new functional sponge. At the time of his original experiments he made the incorrect assumption that the differentiated cells have reverted back to some embryonic type and then re-differentiated following coalescence. This error was first corrected by J. S. Huxley (1911, 1921), who worked with another species of sponge and showed that the different cells retained their differentiation following dissociation. He pointed out that Driesch's dictum, that the fate of a cell is the function of its position, does not hold in this case, but the reverse, for the position of a cell is a function of its



differentiation, that is the differentiated cells wander about in the coalesced clump of dissociated cells until they find their proper location. This point has been confirmed in numerous ways by different workers, all of which is excellently reviewed by P. Brien (1937), who has contributed some evidence himself.

Much the same story holds for the reassociation of coelenterate cells, and more recently there have been some remarkably convincing experiments on dissociated vertebrate cells. In 1952 Weiss & Andres injected dissociated presumptive melanoblasts into the blood-stream of chick embryos and they found that these cells become lodged in their appropriate region in the embryo. Townes & Holtfreter (1955) were able to show such specific reorganization in dissociated amphibian embryos, although the best evidence that the cells retain their differentiations comes from recent work. In particular, Trinkaus (1957) has been able to follow the cell types using isotope markers, and Moscona (1957) has used an elegant method with a mixture of cells from different species. Previously he had developed a method of dissociating cells by the use of trypsin and then to mark his cells he used combinations of chick and mouse cells, each of which is histologically recognizable. If mouse and chick cartilage were mixed, a mass of continuous cartilage resulted in which mouse and chick cartilage-cells were randomly distributed. However, if mouse cartilage-cells were mixed with chick kidney-cells then the cells by migration formed discrete groups of a mass of cartilage and a mass of kidney-tissue. The important proof is that all the cartilage was mouse and all the kidney was chick; there had been no cell transformation but merely a regrouping of the cells.

It should be noted that these experiments of Moscona differ from the slime-mould experiments reported here, in that his cells showed no specificity in their regrouping with respect to species, but only with respect to the tissue. In the slime moulds, the cells show species specificities in their regrouping, but thus far we have not demonstrated any tissue regroupings. If we are to draw a conclusion from this paradox it is perhaps that the common denominator in this interesting process of sorting out or regrouping is not the degree of differentiation, or determination, of the cells, but rather the degree of surface compatibility among the cells. Cells which are completely compatible will mix randomly, cells which are completely incompatible will separate completely, cells which show an intermediate degree of surface compatibility will regroup by sorting out. This intermediate degree of surface compatibility may be produced either by species differences, as in the slime moulds, or differences in cell differentiation, as in vertebrate cells. Moreover, in the case of sponges this incompatibility can be produced both ways, for Galtsoff (1929) showed that in mixtures of cells of different sponges there was a sorting out with respect to differentiation as well as a sorting out with respect to species.



## SUMMARY

The cells of various species and strains of cellular slime moulds have been mixed by making grafts at the aggregation stage and by thoroughly mixing the cells at the migration stage. Depending upon the strains used, varying degrees of cell compatibility or adhesion between the cells was observed. There was either (1) a complete separation of the strains; (2) a partial merger to form a double fruiting body, one standing on the sorus of the other; (3) a single fruiting body on which the pre-spores of both strains regrouped to form two cohesive blocks of cells within one sorus.

This regrouping phenomenon is also characteristic of many dissociated animal cells where partially or completely differentiated cells will regroup with their kind. It is suggested that the basis of this regrouping phenomenon is the result of an intermediate degree of surface compatibility—not so great as to produce a complete separation of the cells, and not so little as to permit a random intermingling of the cells.

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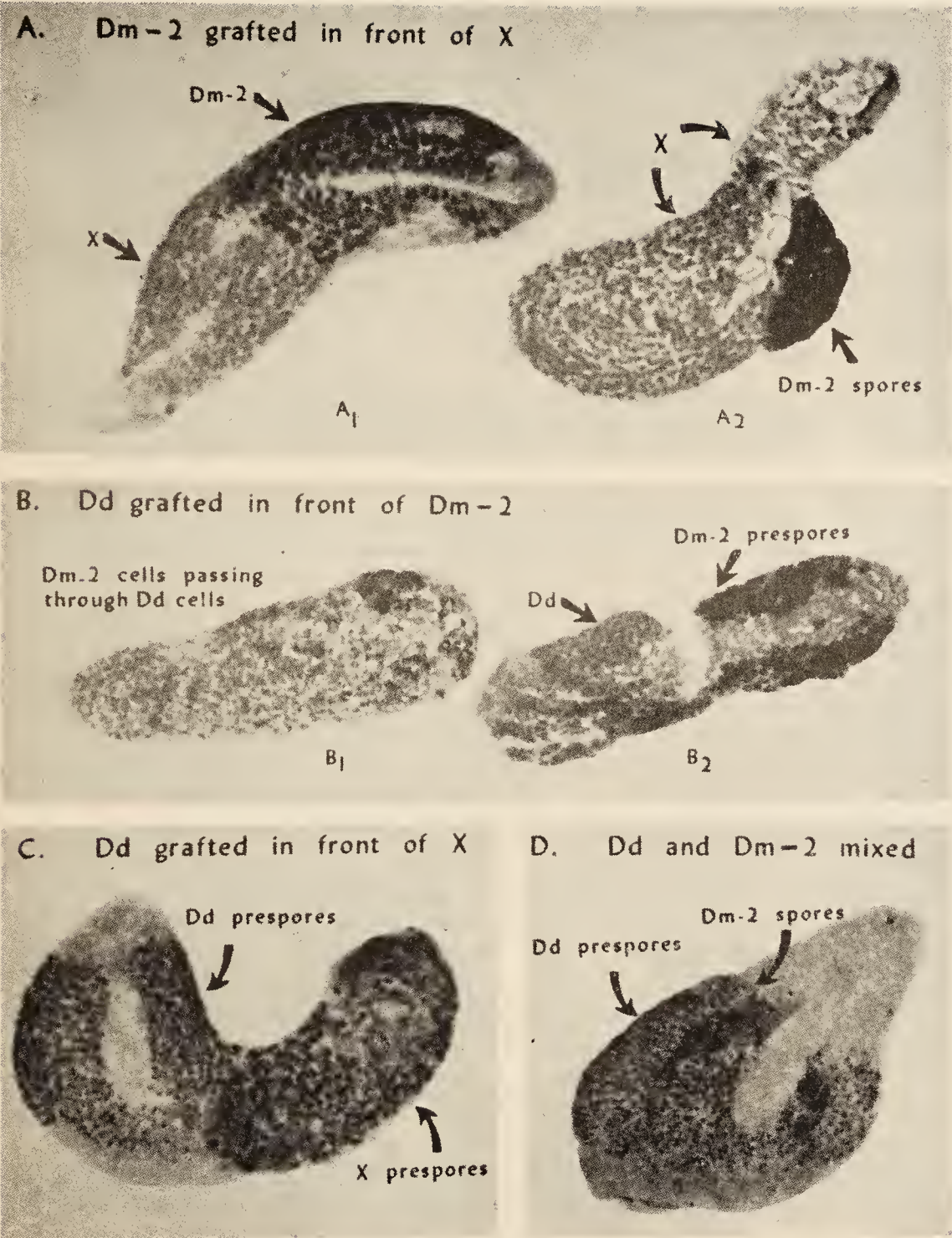
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#### EXPLANATION OF PLATE

Photographs of sections stained by the periodic acid-Schiff method for non-starch polysaccharides. The text should be consulted for details.

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*EVIDENCE FOR THE SORTING OUT OF CELLS IN THE  
DEVELOPMENT OF THE CELLULAR SLIME MOLDS\**

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In the development of the cellular slime molds, independent amoebae stream together to central collection points and the resulting sausage-shaped cell masses will migrate for varying periods of time before forming fruiting structures. In a number of species it is evident during the migration phase that the anterior cells of the mass are the cells which will make up the stalk and the posterior cells develop into the sorus, for each individual amoeba in the hind end becomes encapsulated into a spore.

In a study made some years ago Bonner<sup>1</sup> suggested, on the basis of observations with vital dyes, that the position of a cell within the migrating slug was determined by the position of the cell within the aggregation pattern; if a cell were near the center of the aggregating area it would assume an anterior position in the slug, and if a cell were at the periphery of the aggregating area it would assume a posterior position. It is now clear that the method used was totally inadequate and that there is a major rearrangement or sorting out of the cells during aggregation. The evidence for this conclusion comes from a number of previous studies which will be briefly summarized as well as from new experiments that will be presented here.

Before giving the evidence for cell realignment during the aggregation stage, it should be mentioned that there are some observations which indicate a relatively minor rearrangement in the migration stage. Raper<sup>2</sup> was the first to show that if colorless and vitally stained migrating masses of *Dictyostelium discoideum* were cut in two pieces and grafted reciprocally (so that the anterior half was colored and the posterior half colorless, and vice versa) the division line between the colored and colorless regions remained fairly sharp. In repeating these experiments and watching the grafted cell mass for extended periods of time, Bonner<sup>3</sup> showed that a few individual cells did cross the division line and it was even possible to calculate their rate of forward and backward movement.<sup>4</sup> The number of such especially fast- or slow-moving cells was small in comparison to the total number of cells in

the mass and the phenomenon could not account for any major redistribution of the cells within the mass, except over an extended period of time.

In the above experiments the grafts were made in such a way that the normal positions were retained, that is, anterior fractions were kept in an anterior position and posterior fractions in a posterior position. If, on the other hand, a piece of vitally colored anterior region was added to the posterior end of an intact colorless slug, then this colored section slowly moved forward as a unit, while the whole mass migrated, so that ultimately it assumed an anterior position.<sup>3</sup> It was also true that colored posterior grafts placed anteriorly would eventually assume a posterior position. In the controls where posterior bits were placed in the posterior end and anterior bits in the anterior end, the grafts did not shift their positions. Therefore it appeared that the anterior and the posterior cells differed in their rate of movement and if displaced they would return to their original position within the slug. Since normally the majority of the cells within the mass retained their relative positions, there is the possibility that during aggregation the cells have redistributed so that the fast cells are at the anterior end and the slow cells in the posterior end.

To test the possibility of such cell redistribution during aggregation, grafts using amoebae stained with vital dyes were attempted during the aggregation stage.<sup>5</sup> The center of a colorless aggregation pattern was removed and replaced by a vitally stained center (or vice versa) and if there were no redistribution of cells one would expect the resulting slug to be colored at one end and colorless at the other. However the result invariably gave a slug of uniform coloration indicating a major reshuffling while the cells are aggregating.

During the course of the same experiments,<sup>5</sup> it was also shown that the cells of different species and strains had different rates of movement and that the cells of a fast strain could filter past the cells of a slower strain and accumulate in the anterior end of an artificially created chimaera. It is conceivable that the mechanism of cell rearrangement in these strain and species mixtures might parallel the rearrangement within a single strain, which is the subject of this contribution.

The experiments to be presented below were undertaken with two objectives in mind: one was to obtain further and more conclusive evidence whether or not there was normally a sorting out of the cells during aggregation, and the other was to find if any particular character could be associated with the cells that reached the anterior position as opposed to those that assumed the posterior position in the migrating cell mass.

*Materials and Methods.*—The following slime molds were used for these experiments: *D. discoideum* (DD-1); *D. mucoroides* (DM-11) which includes TYP (or wild type) and two variants of this strain isolated by Filosa<sup>6</sup> which give distinctive abortive fruiting bodies, MV and GV; *Polysphondylium violaceum* (PV-1) which consists of two variants isolated by Filosa,<sup>6</sup> a long and a short migration form. All the slime molds were grown on *Escherichia coli*.

The method for isolating single spores from one sorus was as follows: the sorus was touched with a glass rod possessing a small bead at its tip. The bead was then drawn over the surface of sterile agar in order to spread the spores over a restricted area. Approximately 200 to 250 spores were counted directly on the agar using the high powers of a dissecting microscope (12× oculars, 6× objective). These were



then cut out on an agar block with a small scalpel and placed in 2.0 to 2.5 ml of sterile water (so as to have approximately 100 spores/ml). A loopful of bacteria was added to the tube and after a thorough shaking 0.2 ml was dispensed in each Petri dish of nutrient agar (0.5 per cent peptone, 0.5 per cent dextrose, 2.0 per cent agar). The inoculum drop was then spread over the surface of the agar with a glass rod and the plates were incubated at 18 to 21°C. The growing plaques of amoebae were circled with a glass marking pencil on the bottom of the Petri dish as soon as they appeared and after fruiting their character was recorded and scored.

*Results.—Cell redistribution:* The basic principle of these experiments was to have a mixture of two cell types in an aggregate and then observe if both cell types were present in equal frequency along the axis of the slug or whether each redistributed itself so that it was predominantly at one end. If the distribution were uneven one could conclude that there had been a rearrangement of the cells, for presumably there was a random distribution of the two cell types over the surface of the substratum before aggregation.

The experiment was done in two ways. In one, a strain which consistently held two cell types in a fairly constant ratio was allowed to aggregate and the emerging sausage was cut into three sections, each producing a small fruiting body. The spores of each of these fruiting bodies were then tested for the relative number of the two cell types by scoring the progeny of the spores upon single spore isolation. When this was done with a mixed strain of *D. mucoroides* (strain DM-11) which contains approximately 85 per cent TYP or wild-type cells and approximately 15 per cent MV cells, then the emerging slug showed 36 per cent MV cells in the posterior fraction (based on counts of 170 spores or plaques, and henceforth the numbers in parentheses will indicate the size of the sample), 6 per cent MV cells (149) in the middle fraction, and 1 per cent MV cells (113) in the anterior fraction.

A similar experiment was performed with *P. violaceum*, but in this case, since the normal fruiting body breaks up into a series of small sori jutting off the main axis of the stalk in whorls, it is not necessary to cut the slug into sections; in fact the cell mass normally breaks up into a series of smaller cell masses from which whorls of side branches simulating miniature fruiting bodies develop. If a strain is used (PV-1) which contains cells which produce colonies that either have a long or a short migration character, then again it is possible to test the distribution of these two cell types within the cell mass. In sori from the posterior end, the frequency of the short migration cell type is 35 per cent (280), while the anterior or apical sorus shows a frequency of 5 per cent (472). (These figures are a summary of five separate experiments all of which showed consistent results.) Since the results for both species show different relative amounts of two cell types along the axis of the slug, there is obviously a redistribution of the cells.

In the second type of experiment the vegetative amoebae of the two cell types (MV and TYP of DM-11) were thoroughly mixed with an eyelash cemented on a glass tube. They were then allowed to continue feeding, and approximately 24 hours after mixing aggregation occurred and the resulting slugs were cut into three sections, again each section producing a small fruiting body, the spores of which were tested. Of two cases examined, one showed 97 per cent MV cells (62) in the posterior fraction, 91 per cent (117) in the middle fraction, and 66 per cent (148) in the anterior fraction. In the second case the posterior fraction showed 86 per



cent MV cells (84), the middle fraction was lost, and the anterior fraction showed 67 per cent (131). These results are consistent with the previous set even though the relative number of MV cells is larger.

Also a few grafts were made at the aggregation stage. If the center of a pure wild-type aggregation pattern was replaced by a pure MV center, one composite slug resulted. This was cut into three sections as before, and in one case the posterior cells were all typical (171), the middle fraction showed 52 per cent MV cells (182), and most important, the anterior fraction had 39 per cent MV cells (153). In other words, the anterior position had changed during the course of the latter half of aggregation from 100 to 39 per cent MV cells, representing a considerable redistribution. This was confirmed in another case (in which the middle and

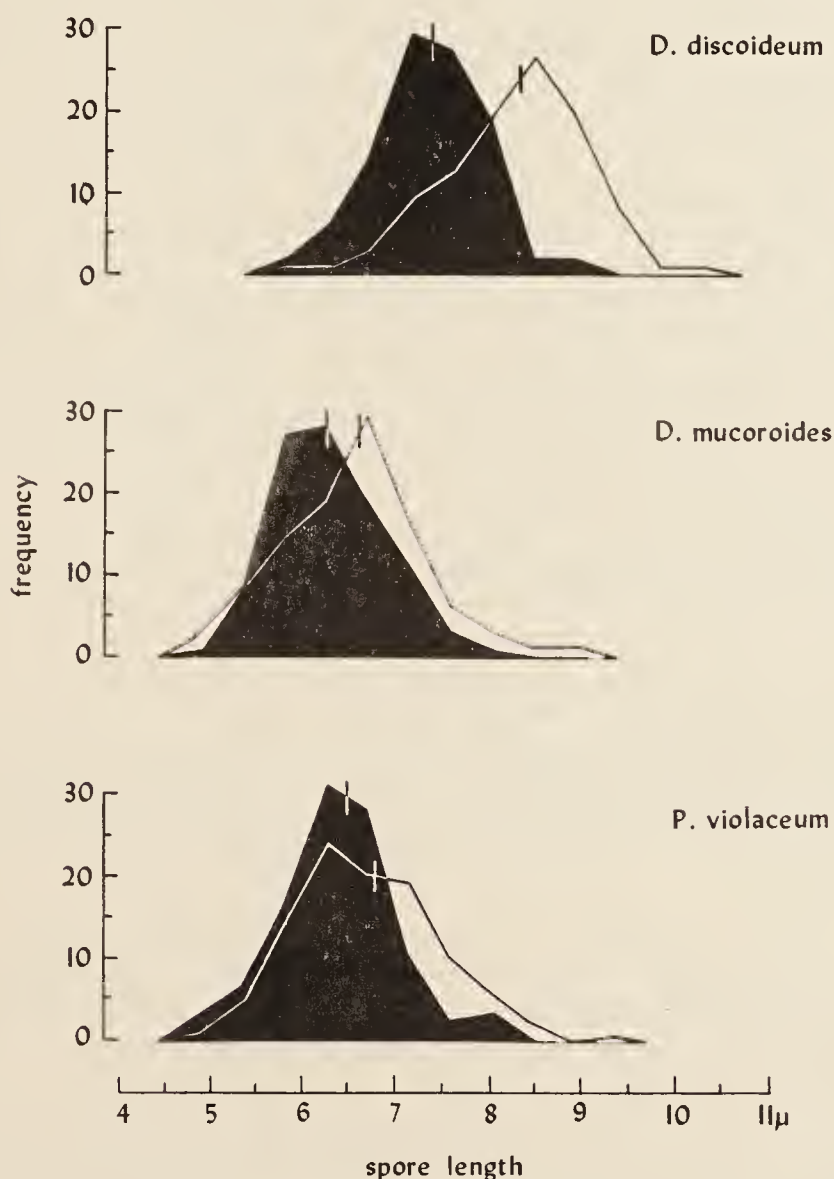


FIG. 1.—Frequency distribution of spore lengths of three species of cellular slime mold showing the difference in size between spores derived from the anterior versus the posterior end of the cell mass. The black curves are of 100 spores derived from the posterior ends, and the white curves are the same for the anterior ends. The vertical markers on the curves show the means.

posterior fractions were lost) where the anterior end went from 100 to 77 per cent MV cells (52).

*A Difference between the Anterior and Posterior Cells.*—If, as has been demonstrated, there is a redistribution of cells during aggregation, it is now reasonable to ask if any particular character can be associated with those cells that assume the anterior position in contrast to those that assume a posterior position.

The most obvious difference to examine is size. In previous studies it was shown that the anterior cells in the slug of *D. discoideum* are larger than the posterior ones<sup>7</sup> but it was always assumed that this difference did not reflect a difference in the dry weight of the cells, but rather a difference in the amount of water in the cells due to different local physiological conditions in the front and hind end of the slug. To bring the cells to the same physiological condition the following method was used: a slug was cut into three sections and the anterior and posterior fractions were allowed to fruit. Then the size of the spores of the sorus from the anterior fraction was compared with that of the sorus from the posterior fraction. (This was done by drawing the elliptical spores with a camera lucida and measuring their short and long axes. After carefully testing various ways of estimating spore size, it was found that the long axis alone was a completely reliable index of size.)

In this way it was possible to show that in *D. discoideum* spores derived from the anterior end of the slug are significantly larger than those derived from the posterior end. This was done on three separate slugs which showed consistent results and, when combined, gave a total sample of 200 spores for both sori. The mean of the spores derived from the posterior end is  $7.3\ \mu$ , while that of the spores derived from the anterior end is  $8.3\ \mu$ , which indicates a clear difference in the size of the population (Fig. 1). (Using the "student" test, the difference gives a  $t$  value of 12.4, with a  $P$  of  $> 0.01$ .)

These measurements were taken from slugs that had migrated approximately 1 cm. By comparison with spores of slugs that had migrated 5 or more cm, it was possible to show that spore size decreases with continued migration. This was interpreted as supporting the assumption that spore size is a reliable index of the dry mass of a cell, for it would be expected that the energy reserves, and therefore the cell dry mass, would be reduced with prolonged migration.

Comparisons of the spores derived from the front and hind end of a slug were also tested on *D. mucoroides* (wild-type clone of strain DM-11). In one experiment, measuring 100 spores for each sorus, it was again shown that the anterior spores were significantly larger. (Difference by the "student" test gives a  $t$  value of 8.2, the  $P$  being  $< 0.01$ .)

Using *P. violaceum*, which has a compound fruiting body, a posterior and an apical sorus were removed and their spore sizes compared. As in the other species, the apical sorus contained spores that were clearly larger. (Difference based on a sample of 100 spores each gives a  $t$  value of 3.4 the  $P$  being  $< 0.01$ ).

*Discussion.*—These experiments support the notion that during the process of aggregation of the cellular slime molds the larger cells sort out so as to assume an anterior position in the migrating cell mass, while the smaller cells assume a posterior position. It would appear that size is directly correlated with speed of movement, although it is possible that selective adhesion might also play a role. It is

hoped that in the near future it will be possible to test further the relation of size to the rate of movement of an individual cell.

If such a sorting out exists during aggregation, there is the interesting corollary that the sorting out itself will produce a gradient of cell size within the mass. The chemotaxis mechanism of aggregation gives a direction or polarity to the cell movement, and since the cells redistribute with respect to size, the inevitable result is a slug possessing a gradient. One could then assume that such a gradient were the basis of the future differentiation of the anterior pre-stalk cells and the posterior pre-spore cells.<sup>5</sup>

Cellular slime molds are sufficiently unusual in so many respects that it always remains doubtful if the lessons we learn from them have any application to other forms. In the development of many organisms there are mass directed morphogenetic movements of cells, however, and it would be of great interest to know if these movements also involve a sorting out of cells which might provide a stepping stone for the subsequent process of differentiation. There is excellent recent evidence of cell sorting in disaggregated tissues, but its role in normal morphogenesis remains virtually unexplored.

*Summary.*—From previous work there are suggestions that during the aggregation of cellular slime molds there is a redistribution of cells and the more rapidly moving cells assume an anterior position in the resulting slug, while the slower cells lie posteriorly. To test the possibility of cellular redistribution, two compatible cell types were mixed and, although their initial distribution must have been random, their eventual distribution in the slug was markedly non-random. Furthermore, it was found that if a slug were cut up into sections and each fraction allowed to fruit, the spores from anterior fractions were larger than those from posterior fractions. From this it was concluded that normally, during aggregation, there is a sorting out of cells and that the faster-moving large cells are found in higher concentration at the front end of the slug while the smaller cells accumulate at the hind end.

\* The early phases of this study were supported in part by a grant from the National Science Foundation and in part by funds from the Eugene Higgins' Trust Fund allocated to Princeton University. The latter part was done under the auspices of a Guggenheim Fellowship and I would like to express my gratitude to Professor C. H. Waddington for his kind hospitality during my stay at his laboratory. Also I should like to thank Miss Kathleen Dodge for technical assistance during the first phases of the project, and Dr. B. Woolf for his help with the statistics.

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<sup>2</sup> Raper, K. B., *J. Elisha Mitchell Sci. Soc.* **56**, 241–282 (1940).

<sup>3</sup> Bonner, J. T., *Am. Nat.*, **86**, 79–89 (1952).

<sup>4</sup> Bonner, J. T., *Quart. Rev. Biol.*, **32**, 232–246 (1957).

<sup>5</sup> Bonner, J. T., and M. S. Adams, *J. Embr. Exper. Morph.*, **6**, 346–356 (1958).

<sup>6</sup> Filosa, M. F., Ph.D., Thesis, Princeton University (1958).

<sup>7</sup> Bonner, J. T., and E. B. Frascella, *Biol. Bull.*, **104**, 297–300 (1953).



# Differentiation in Social Amoebae

*Certain amoebae gather to form a mass of spores and a stalk. The way in which spore cells and stalk cells segregate may shed light on how the cells of many-celled organisms differentiate into various types*

by John Tyler Bonner

Recently I was asked to talk to two visiting Russian university rectors (both biologists) about the curious organisms known as slime molds. Communication through the interpreter was somewhat difficult, but my visitors obviously neither knew nor really cared what slime molds were. Then, without anticipating the effect, I wrote on the blackboard the words "social amoebae," a title I had used for an article about these same organisms some years ago [see "The Social Amoebae," by John Tyler Bonner; *SCIENTIFIC AMERICAN*, June, 1949]. The Russians were electrified with delight and curiosity. I described how individual amoebae can come together under certain conditions to form a multicellular organism, the cells moving into their appropriate places in the organism and differentiating to divide the labor of reproduction. Soon both of my guests were beaming, evidently pleased that even one-celled animals could be so sophisticated as to form collectives.

Of course there are other reasons why slime molds hold the interest of biologists. The transformation of free-living, apparently identical amoebae into differentiated cells, members of a larger organism, presents some of the same questions as the differentiation of embryonic cells into specialized tissues. In the budding embryo, moreover, cells go through "morphogenetic movements" which seemingly parcel them out to their assigned positions in the emergent organism. The only difference is that the simplicity of the slime molds provides excellent material for experiments.

The slime-mold amoebae, inhabitants of the soil, do their feeding as separate, independent individuals. Flowing about on their irregular courses they engulf bacteria, in the manner of our own

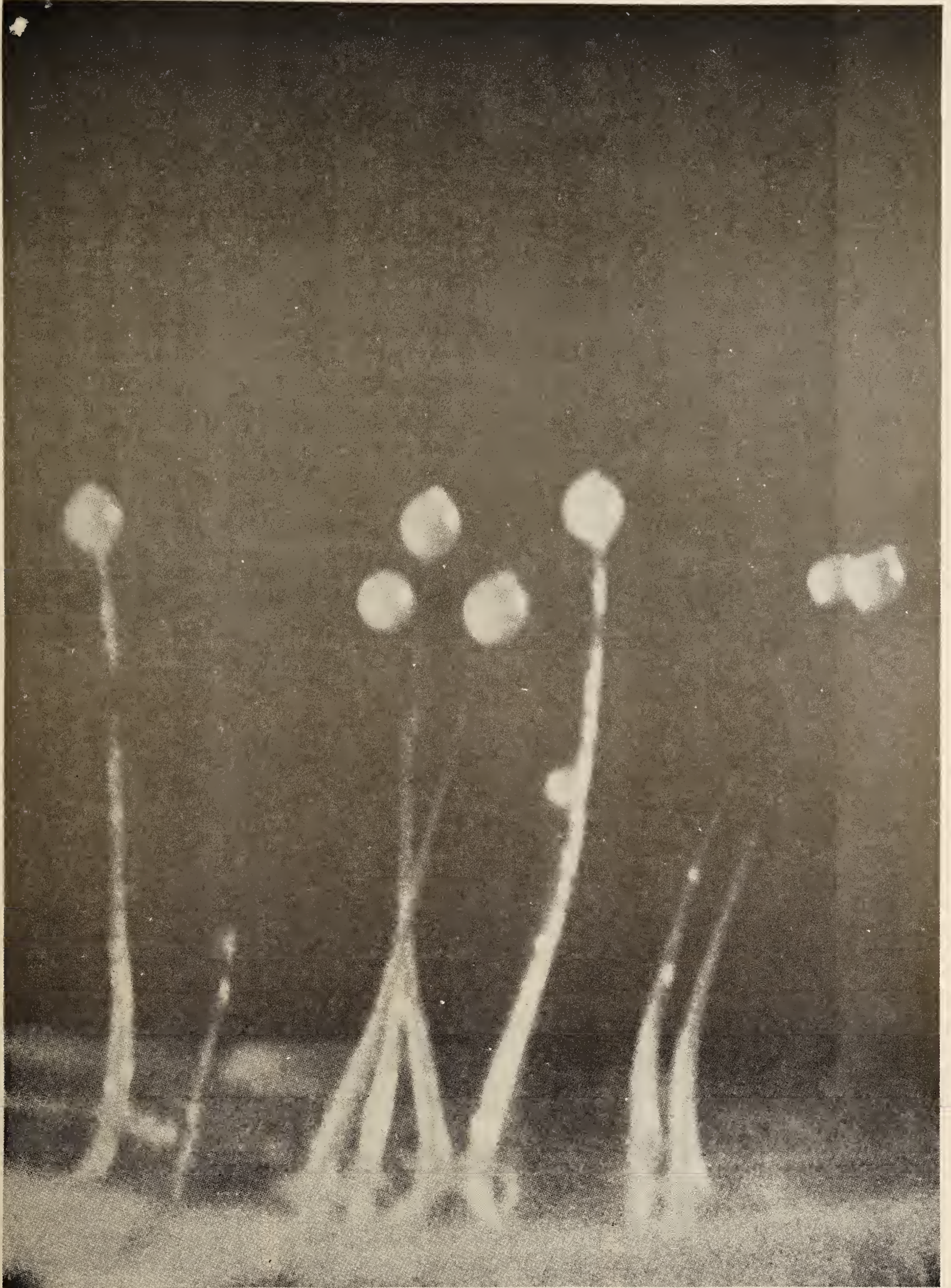
amoeboid white blood cells. At this stage they reproduce simply by dividing in two. Once they have cleared the food away, wherever they are fairly dense, the amoebae suddenly flow together to central collection points. There the cells, numbering anywhere from 10 to 500,000, heap upward in a little tower which, at least in the species *Dictyostelium discoideum*, settles over on its side and crawls about as a tiny, glistening, bullet-shaped slug, .1 to two millimeters long. This slug has a distinct front and hind end (the pointed end is at the front) and leaves a trail of slime as it moves. It is remarkably sensitive to light and heat; it will move toward a weak source of heat or a light as faint as the dial of a luminous wrist watch. As the slug migrates, the cells in the front third begin to look different from the cells in the two thirds at the rear. The changes are the early signs of differentiation; eventually all the hind cells turn into spores—the seeds for the next generation—and all the front cells cooperate to make a slender, tapering stalk that thrusts the mass of spores up into the air.

To accomplish this transformation the slug first points its tip upward and stands on end. The uppermost front cells swell with water like a bit of froth and become encased in a cellulose cylinder which is to form the stalk. As new front cells arrive at the frothy tip of the stalk they add themselves to its lengthening structure and push it downward through the mass of hind-end cells below. When this process, like a fountain in reverse, has brought the stalk into contact with the surface, the continued upward migration of pre-stalk cells heightens the stalk lifting the presumptive spore cells up into the air. Each amoeba in the spore mass now encases itself in cellu-

lose and becomes a spore. The end result is a delicate tapering shaft capped by a spherical mass of spores. When the spores are dispersed (by water or by contact with some passing creature such as an insect or a worm), each can split open to liberate a tiny new amoeba.

What mechanism brings the independent slime-mold amoebae together in a mass? More than a decade ago we found that they are attracted by the gradient of a substance which they themselves produce. In our early experiments we were unable to obtain cell-free preparations of this substance (which we named acrasin); cells actively secreting it were always necessary to start an aggregation. Later B. M. Shaffer of the University of Cambridge got around this barrier in an ingenious experiment. He took water that had been near acrasin-producing cells (but was itself free of cells) and applied it to the side of a small agar block placed on top of some amoebae. The amoebae momentarily streamed toward the side where the concentration of acrasin was higher. Shaffer found that the water must be used immediately after it is collected in order to achieve this effect, and that it must be applied repeatedly. He therefore concluded that acrasin loses its potency rapidly at room temperature. The loss of potency, he showed, is caused by enzymes that are secreted by the amoebae along with acrasin; when he filtered the fluid through a cellophane membrane to hold back the large enzyme molecules, he was able to secure a stable preparation of acrasin. Presumably the enzymes serve to clear the environment of the substance and so enhance the establishment of a gradient in the concentration of acrasin when it is next secreted. Maurice Sussman and his





**SPHERICAL MASSES OF SPORES** of the social amoeba *Dictyostelium discoideum* are held aloft by stalks composed of other

amoebae of the same species. When the spores are dispersed, each can liberate a new amoeba. The stalks are about half an inch high.





AGGREGATING AMOEBAE of *Dictyostelium discoideum* move in thin streams toward central collection points. Each of the centers

comprises thousands of cells. This photograph and facing one were made by Kenneth B. Raper of the University of Wisconsin.

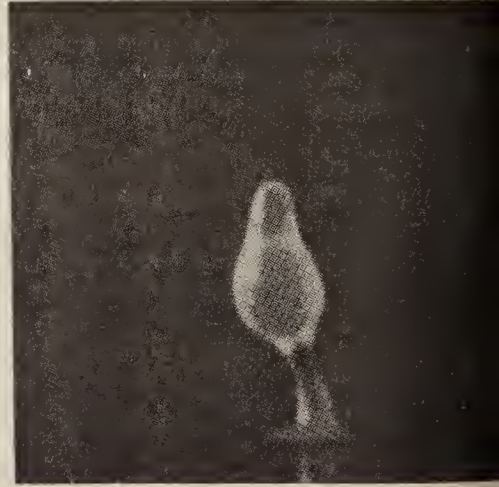
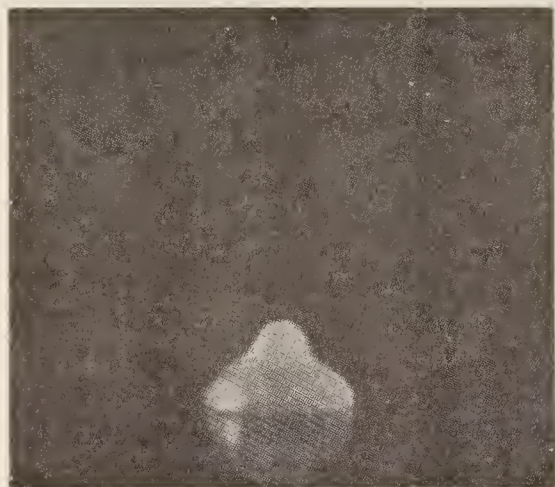
co-workers at Brandeis University in Waltham, Mass., have confirmed Shaffer's work and are now attempting the difficult task of fractionating and purifying acrasin, steps leading toward its identification.

Meanwhile Barbara Wright of the National Institutes of Health in Bethesda dropped a bombshell. She discovered that urine from a pregnant woman could attract the amoebae under an agar block just as acrasin does. The active compo-

nents of the urine turned out to be steroid sex hormones. This does not necessarily mean that acrasin is such a steroid. Animal embryologists were thrown off the track for years when they found that locally applied steroids induce the further development of early embryos. Only after much painful confusion did it become clear that steroids do not act directly on the embryo, but stimulate the normal induction substance. We must therefore consider the

possibility that the steroids act in a similarly indirect manner on the amoebae. The purification of acrasin will, we hope, soon settle the question.

From observations of the cells during aggregation, Shaffer has come to the interesting conclusion that the many incoming amoebae are not responding to one large gradient of acrasin but to relays of gradients. That is, a central cell will release a puff of acrasin that produces a small gradient in its immediate



DEVELOPMENT OF THE FRUITING BODY of a slime mold is shown in this series of photographs made at half-hour intervals.

At far left the tip cells are starting to form a stalk. In the next two pictures the stalk has pushed down through the mass to the





**MIGRATING SLUGS** of *Dictyostelium discoideum* leave trails of slime behind them as they move. The photographs in this article

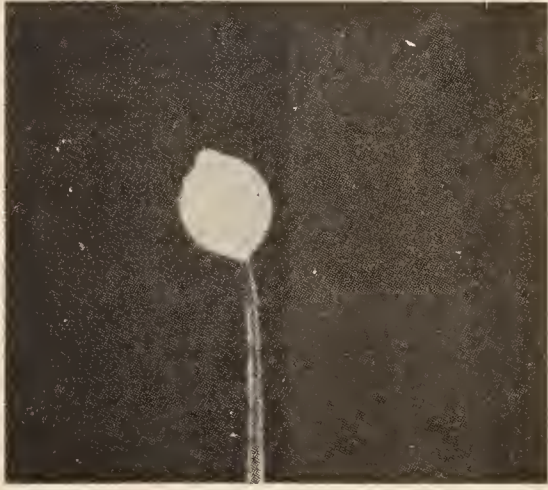
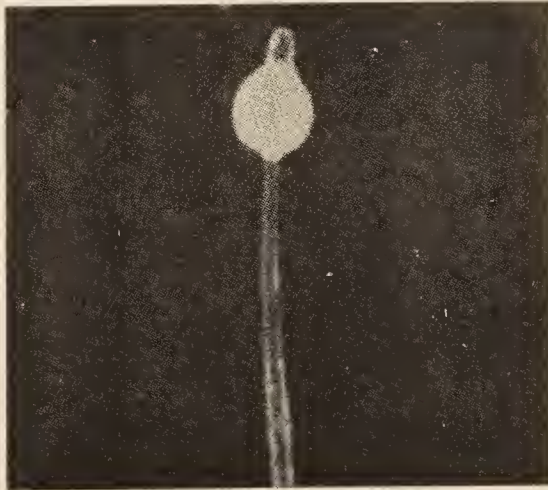
appear in *The Cellular Slime Molds*, by John Tyler Bonner, and are reproduced with permission of Princeton University Press.

vicinity. The surrounding cells become oriented, and now produce a puff of their own. This new puff orients the cells lying just beyond, and in this way a wave of orientation passes outward. Time-lapse motion pictures show the amoebae moving inward in waves, which could well represent the relay system. If this interpretation is sound, then the rapid breakdown of acrasin by an enzyme plainly serves to clear the slate after each puff in preparation for

the next. The cells do not depend entirely on acrasin for orientation; once they are in contact they tend to stick to one another and the pull-tension of one guides the cells that follow. This is a special case of contact guidance, a phenomenon well known in the movements of embryonic cells of higher animals.

After the amoebae have gathered together, what determines their position within the bullet-shaped slug?

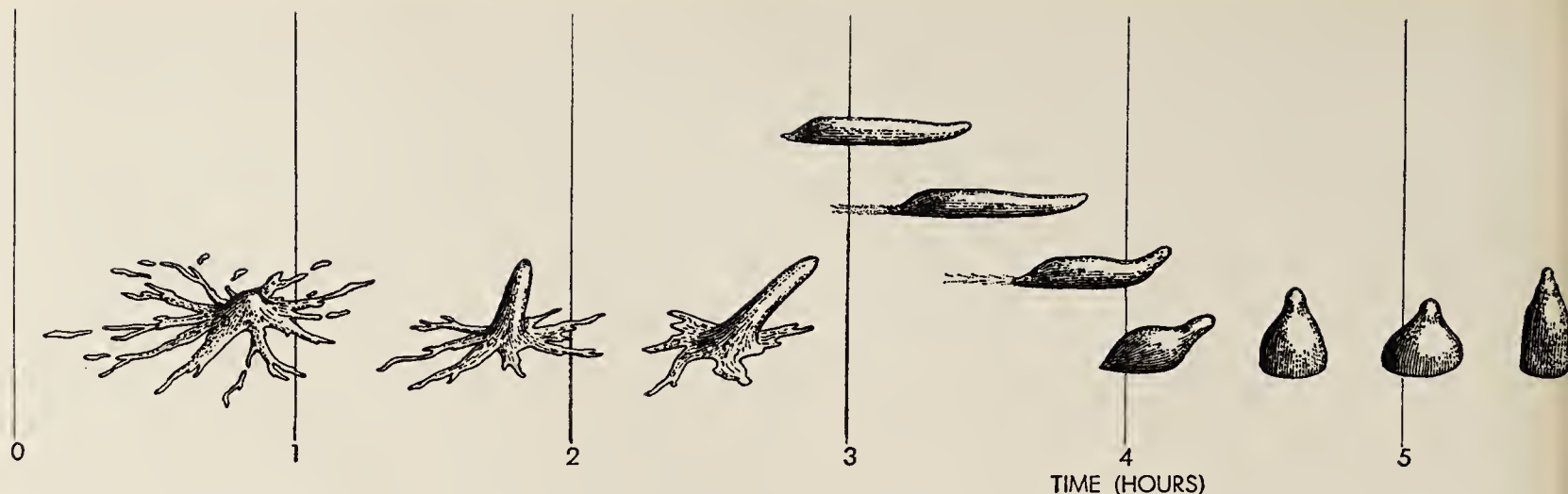
One might assume that the cells that arrive at the center of the heap automatically become the tip of the slug, and that the last cells to come in from the periphery make up the hind end. If this were the case, chance alone would determine whether a cell is to become a front-end cell and enter into the formation of the stalk, or a hind-end cell and become a spore. If, on the other hand, the cells rearrange themselves as they organize into a slug, then it is conceivable



surface and is starting to lift the cell mass. In the fourth picture the spores have formed their cellulose coats, making the ball more

opaque. In the last two pictures the spore mass moves up to the very top of the stalk, as the stalk itself becomes still longer.





**LIFE CYCLE OF A SLIME MOLD**, typified by *Dictyostelium discoideum*, involves the aggregation of free-living amoebae into

a unified mass (*first three drawings*), then the formation of a slug which moves about for a time (*next four drawings*) and finally

able that the front end might contain selected cells, differing in particular ways from those in the hind end. I am embarrassed to say that in 1944 I presented some evidence to support the idea that their chance position was the determining factor—evidence that, as will soon be clear, was inadequate. It is some comfort, however, that I was able to rectify the error myself.

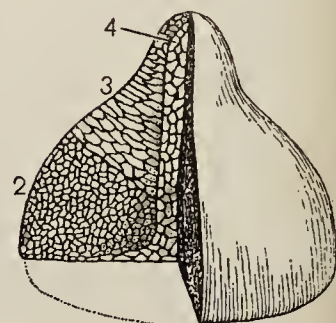
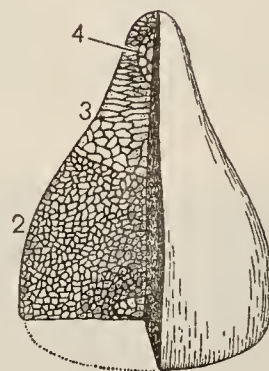
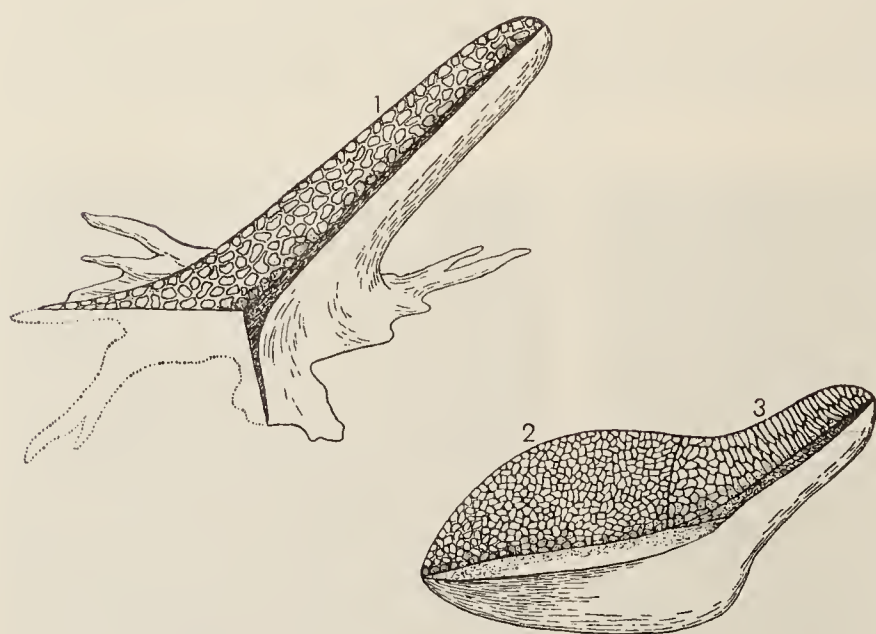
The first faint hint that the cells do redistribute themselves in the slug stage came when we repeated some experiments first done by Kenneth B. Raper of the University of Wisconsin. We stained some slugs with harmless dyes and then grafted the hind half of a colored slug onto the front half of an unstained slug. The division line remained sharp for a

number of hours, just as Raper had previously observed. But later we noticed that a few stained cells were moving forward into the uncolored part of the slug. In the reverse graft, with the front end stained, a similar small group of colored cells gradually migrated toward the rear end of the slug. Still, the number of cells involved was so small that it could hardly be considered the sign of a major redistribution. Next we tried putting some colored front-end cells in the hind end of an intact slug. The result was a total surprise: now the colored cells rapidly moved to the front end, traveling as a band of color up the length of the slug.

Here was a clear demonstration that the cells do rearrange themselves in the

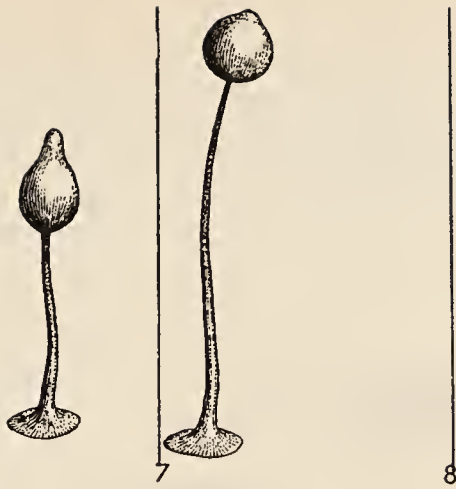
slug and that there is a difference between the cells at the front and hind ends. The difference between front-end and hind-end cells—whatever its nature—was confirmed in control experiments in which we grafted front-end cells to the front ends of the slugs and hind-end cells to the hind ends; in each case the cells maintained their positions.

It looked as if front-end cells were selected by their speed; the colored cells simply raced from the rear end to the front. When we placed hind-end cells in the front end, they traveled to the rear, outpaced by the faster-moving cells, which again assumed their forward positions. We tried to select fast cells and slow cells over a series of genera-



**CUTAWAY DRAWINGS** of five stages show how the cells change. At the end of aggregation all cells appear the same (1), but in the

slug they are of two types (2 and 3). The cells near the tip (3) gradually turn into stalk cells (4) and move down inside the



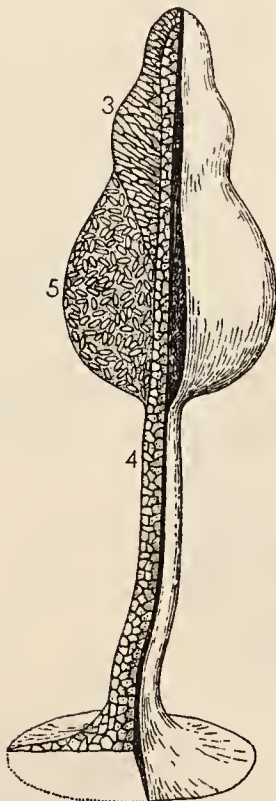
the development of a fruiting body (*last six drawings*). Times are only approximate.

tions to see if speed was a hereditary trait, but after selection the cultures showed no differences from one another or from the parent stock.

Quite by accident a new bit of evidence turned up in an experiment designed for totally independent reasons. Instead of using the fully formed slug we stained amoebae colonies in the process of aggregation and made grafts at this stage by removing the center of the stained group and replacing it with a colorless center, or vice versa. In either case the resulting slug was always uniformly colored, indicating a rapid reasortment of the cells during the formation of the slug.

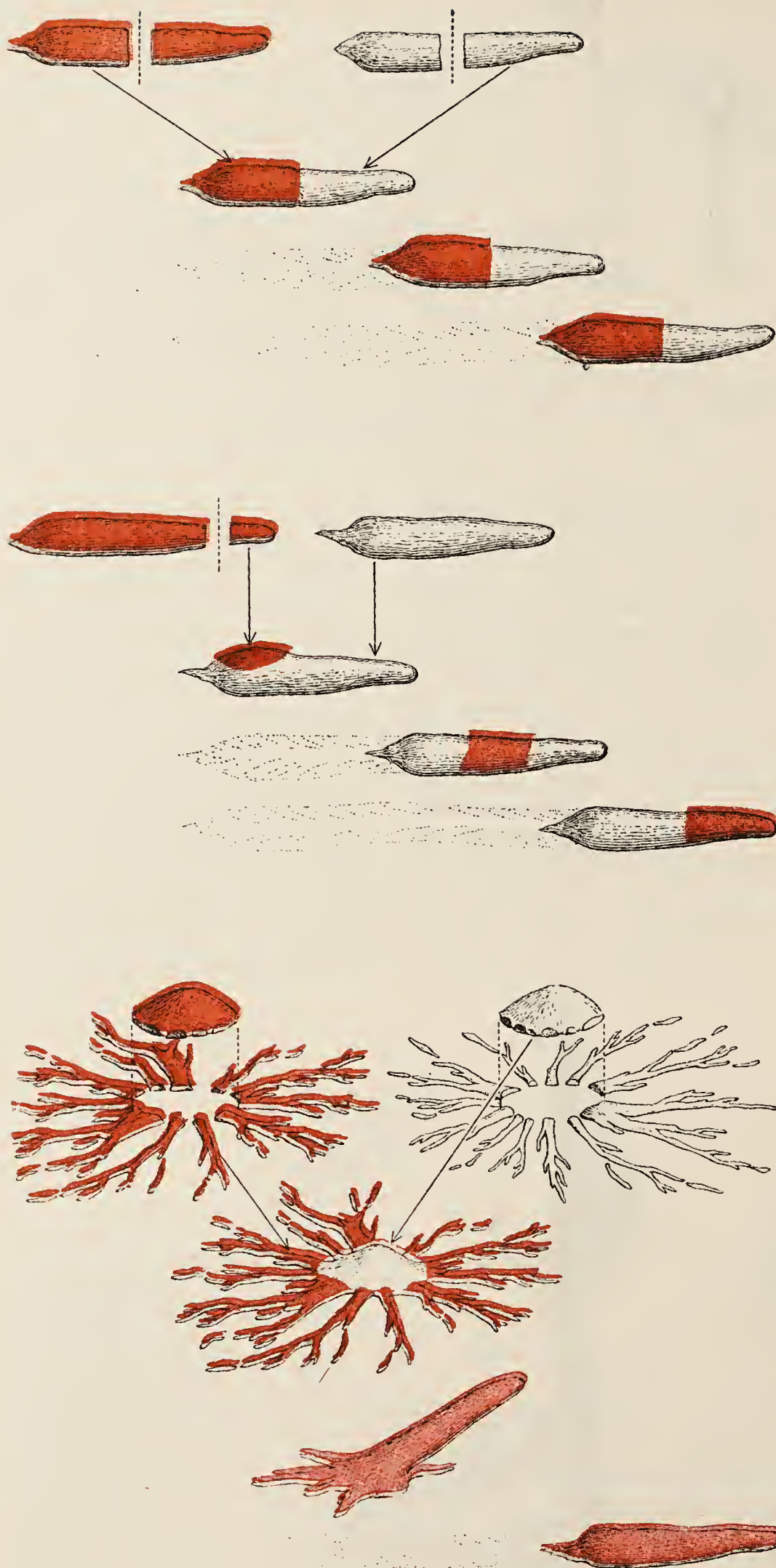
The evidence for a rearrangement of

c



mass. The others (2) become spores (5) as the growing stalk lifts them into the air.





COLORED AGGREGATE in which the center has been replaced by a colorless center produces a uniformly colored slug, indicating that the cells are rearranged as the slug forms. The experiment illustrated in these drawings was originally performed by Kenneth B. Raper.

cells was becoming impressive, but I felt uneasy about the reliability of tests with dyes because such tests had led me into my earlier error. We needed to confirm our results by a different method.

At about this time M. F. Filosa, who was working in our laboratory on his doctoral dissertation, discovered that many of our amoeba cultures contained more than one genetic type. By isolating and cultivating single cells of each type he was able to obtain pure strains that displayed various recognizable abnormalities—in the way they aggregated, in the shape of their slugs or in the form of their spore masses [see illustration on page 160]. The discovery of these strains furnished natural “markers” for identifying and following cells.

Of course there remained one technical problem: How could the individual cells be identified? Fortunately Raper had shown some time earlier that each fragment of a slug that has been cut into pieces will form a midget fruiting body. Spores derived from the several fragments can then be cultured individually. The amoeba from each spore will give rise to many daughter amoebae which can be scored for mutant or normal characteristics as they proceed to form slugs and fruiting bodies.

In one experiment we started with a culture of cells in the free-living feeding stage, into which was mixed 10 to 15 per cent of mutant cells. If we were to find a higher concentration of one type of cell in one part of the resulting slug, then we could conclude that there had been a rearrangement. We allowed the cells to form a slug and cut it up into three parts. Upon culturing the individual spores produced by each part, we found that the hind third had 36 per cent mutant cells, the middle third 6 per cent and the front third 1 per cent. Nothing could be more clear-cut; obviously the cells sort themselves out in a way that brings the normal cells to the front end of the slug. In another experiment, with a larger percentage of mutant cells in the mixture, hind and middle fractions contained 91 per cent mutant cells, and the front end only 66 per cent. Further experiments, including some with other species of slime mold, all led to the same conclusion. During the process of slug formation some cells are more likely to reach the front end than others, and the position of a cell in the slug does not merely depend upon its chance position before aggregation.

One must assume that certain cells move to the front because they travel the



fastest, while the other, slower cells are left behind in the rear end of the slug. Considering the different fates of the front and rear cells, however, it is natural to wonder whether there are any other discernible differences between the front and hind cells. Size is one of the easiest qualities to measure, and comparison of spores from the front and rear portions showed that cells of the front segment are larger. From this it might be concluded that the fastest cells are the largest. But size is related to many other factors; some evidence indi-

cates that cells in the front end divide less frequently than those in the hind regions, and this could affect their size. The possibility of a correlation between size and speed can only be settled by further experiment and observation.

**B**ut one fact is inescapable. The cells that tend to go forward are not identical with those that lag behind. Do the differences ultimately determine which cells become stalk cells and which will be spores? The most obvious deduction is that among feeding amoebae

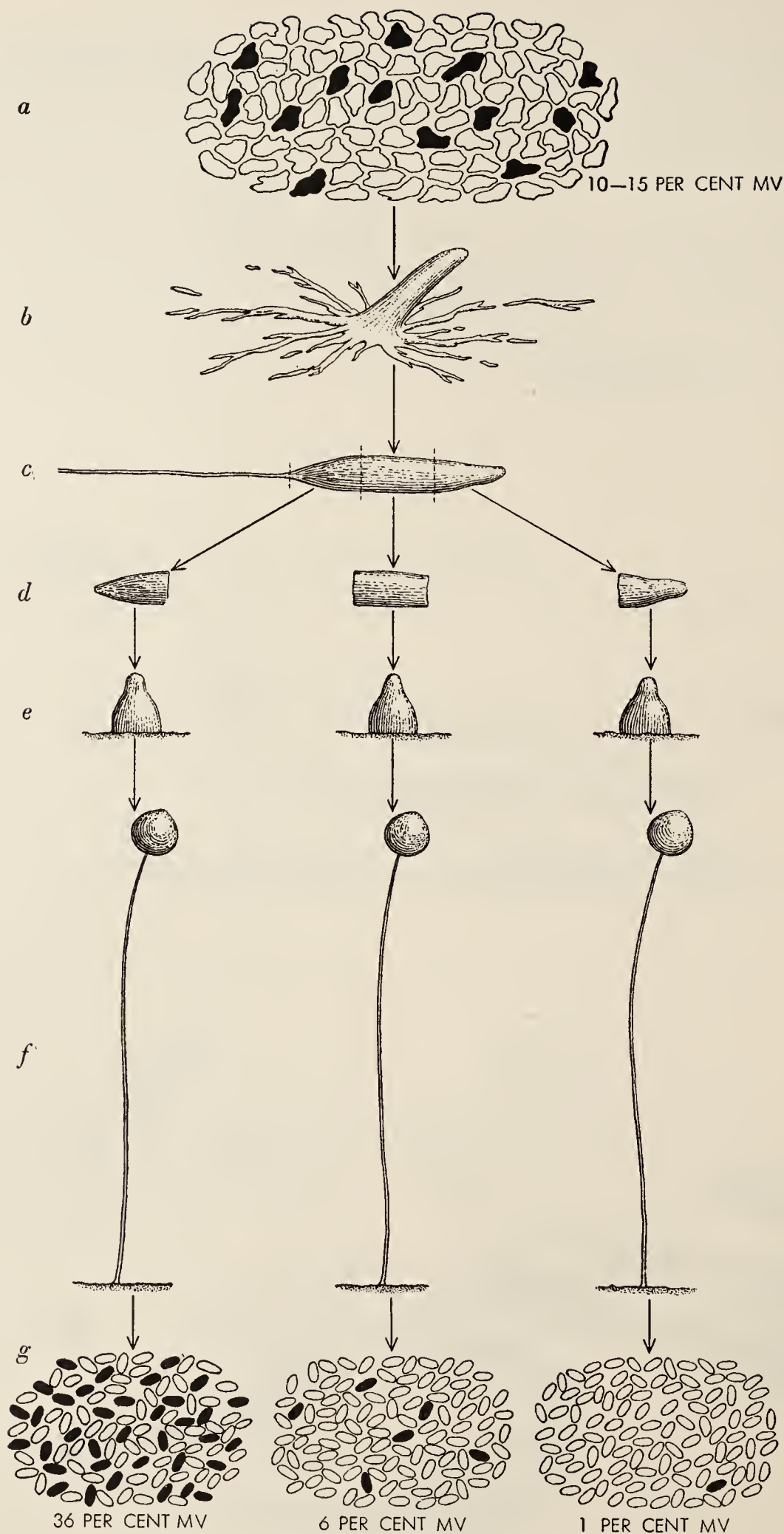
roughly a third are presumptive stalk cells, and the rest are predestined to be spores. This interpretation is clearly false, however, because then it would be impossible to explain how a single fragment of a cut-up slug can produce a perfect miniature fruiting body. The cells in the hind piece, which would normally yield spores, recover from the surgery that isolates them from the large slug, and one third of these presumptive spore cells proceed to form the midget stalk. This remarkable accommodation to a new situation is also exhibited by



**NORMAL AND MUTANT STRAINS** of *Dictyostelium mucoroides* are contrasted in these drawings. The normal form (a) aggregates in thin streams, and its slug remains anchored by a thin stalk. The

“MV” mutant (b) aggregates in broad streams and produces a starfish-like slug which then breaks up into smaller slugs. The stalk of the mutant is usually shorter than that of normal strain.





**REDISTRIBUTION OF CELLS** was proved in an experiment in which MV mutant cells (*black*) were randomly mixed with normal cells at feeding stage (*a*). The cells aggregated (*b*), and the resulting slug (*c*) was cut into three parts (*d*). Each part produced a fruiting body (*e* and *f*). Spores of each were then identified (*g*) by culturing them separately. The concentration of mutant cells was markedly higher in spores from the hind part of the slug.

many types of cells in embryos and in animals capable of regenerating limbs and organs.

A more reasonable way to explain the relation between sorting-out and differentiation is to visualize the aggregating amoebae as having all shades of variation in characteristics between the extremes found at the ends of the slug. As they form a slug the cells place themselves in such an order that from the rear to the front they display a gradual increase in speed, in size and perhaps in other properties not yet measured. Thus each fragment of a cut-up slug retains a small gradient of these properties. It is conceivable that the gradient, set up in the process of cell rearrangement, actually controls the chain of events that leads the front cells to form a stalk and the hind cells to become spores. For the present, however, this is only conjecture.

At this point let me emphasize that the sorting-out process is not unique to slime molds. Recently A. A. Moscona of the University of Chicago and others have found that if the tissues of various embryos or simple animals are separated into individual cells, the cells can come together and sort themselves out [see "Tissues from Dissociated Cells," by A. A. Moscona; *SCIENTIFIC AMERICAN*, May]. For instance, if separate single pre-cartilage cells are mixed with pre-muscle cells, the cartilage cells will aggregate into a ball and ultimately form a central mass of cartilage surrounded by a layer of muscle. By marking the cells in a most ingenious way Moscona showed that there was no transformation of pre-cartilage cells into muscle cells or vice versa; each cell retained its original identity but moved to a characteristic location. In animals, then, sorting-out appears to be a general phenomenon when the cells are artificially dissociated. Since the movement in slime molds is part of their normal development, this raises the challenging question whether such sorting-out occurs in the normal development of animal embryos as well.

One must concede that slime-mold amoebae do profit by collectivization: the aggregate can do things the individuals cannot accomplish alone. In the amoebae's society, however, all are not created equal; some rise to the top and others lag behind. And then there is this distressing moral: Those that go forward with such zest to reach the fore are rewarded with sacrifice and destruction as stalk cells. It is the laggards that they lift into the air which survive to propagate the next generation.



## A NOTE ON SPORE GERMINATION IN THE CELLULAR SLIME MOLD *Dictyostelium mucoroides*<sup>1</sup>

GEORGE KEITH RUSSELL<sup>2</sup> AND JOHN TYLER BONNER

There are a few references to conditions affecting germination in the cellular slime molds in the older literature. Cienkowski (1873) reported that water alone was sufficient for germination in *Guttulina* and Skupienski (1920) obtained germination of *Dictyostelium mucoroides* in distilled water. Olive (1920) on the other hand claimed that a nutrient medium was necessary for germination in this species and this view was independently supported by Potts (1902) who reported no germination in distilled water and 10 per cent germination in tap water. Furthermore he obtained excellent germination in Knop's solution ( $\text{KNO}_3$ ,  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{K}_3\text{PO}_4$ , and  $\text{MgSO}_4$ ), and presented evidence that the phosphate in the solution was of key importance. Because of this conflicting information, some more detailed experiments were carried out, following the rules and procedures of spore germination tests set down by McCallan and Wilcoxon (1932).

**Materials and methods.** *Dictyostelium mucoroides*, strain No. 11 was used in all the tests, and it was grown on *Escherichia coli*.

Two different media were employed: a) standard medium containing 10 gm. of peptone, 10 gm. of dextrose, 0.96 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1.45 gm. of  $\text{KH}_2\text{PO}_4$ , 20 gm. of agar, and 1000 ml. of distilled water; b) non-nutrient agar containing the same buffers listed above and 20 gm. of Difco Bacto agar in 1000 ml. of distilled water.

The spores were prepared by removing several sori from stock cultures of known age with an innoculating loop and suspending the spores in 2 ml. of distilled water. After vigorous shaking (sufficient to disperse any clumps) 0.2 ml. of the suspension was pipetted onto the surface of the bacteria-free medium in Petri plates. The suspension was spread over the surface of the agar with a bent glass rod. After 15 minutes the plates were inverted to drain off the excess water and placed (in a cannister to reduce evaporation) in a 22°C incubator.

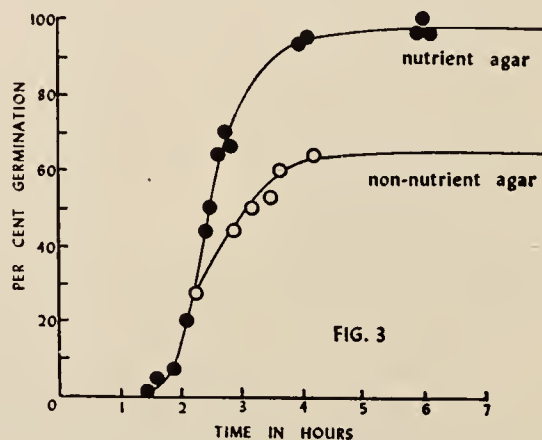
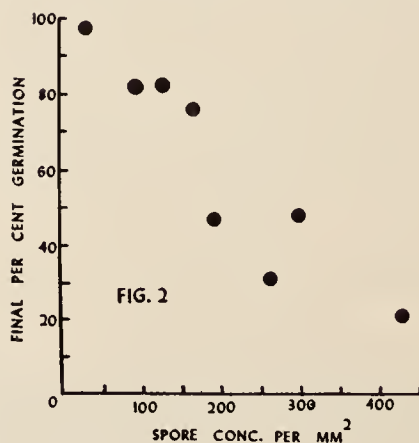
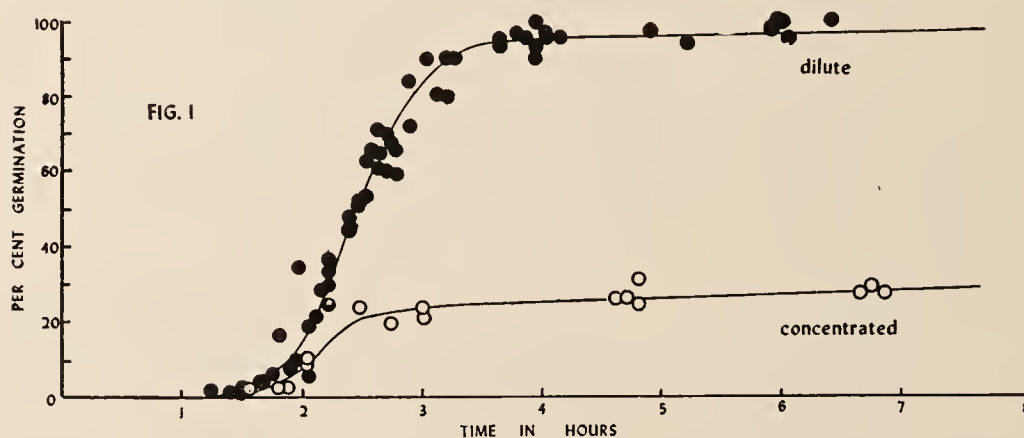
It was found very important to use fresh Petri plates and take every possible precaution to reduce water loss as old dry medium would seriously inhibit spore germination.

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At specific time intervals sections of the agar were punched out with the open end of a test tube, placed on a glass slide and covered with a No. 1 coverslip. The preparation could then be examined (at 640 $\times$  magnification) with a phase microscope possessing a high focal point condenser. In this way it was possible to distinguish between the ungerminated spores and the empty germinated spore cases. For each disc at least 100 spores or the empty spore cases were counted and scored.

In the experiments in which the spore density was determined, it was found that despite all possible care, there was some variation in the density in different parts of the plates. The density given is an average number of counts and the standard errors are given in table 1. These counts were made by scoring the total number of spores in a field with the microscope



FIGS. 1-3. FIG. 1. Graph showing the per cent germination at different times for concentrated and dilute suspensions of *D. mucoroides* spores. FIG. 2. Graph showing the relation of final per cent spore germination to the density of the spores on the agar surface. FIG. 3. A comparison of the germination curves of spores placed on nutrient and non-nutrient agar.

at 400 $\times$  magnification, that is, the total number of spores in an area of .12 mm.<sup>2</sup>

**Results.** Germination is conveniently represented as per cent plotted against time (Fig. 1). When conditions of maximum moisture were maintained, and when the spore density was less than 33 spores/mm<sup>2</sup>, then on nutrient medium the germination almost reaches 100 per cent after about four hours (Fig. 1, upper curve). This particular experiment was repeated seven times with essentially identical results.

**The effect of spore concentration:** If, under similar circumstances, the spore density is greatly increased, then the initiation of germination occurs at about the same time, but after about 4 hours the per cent germination levels off at a much lower value. In other words spore germination is inhibited when the spore density is high.

To further explore the relationship between spore density and the per cent germination after the leveling off has occurred (measurements taken between 5-6 hours) these were plotted one against the other (Fig. 2, table 1). There is almost a linear inverse relation between spore density and per cent germination.

TABLE 1

Concentration of spores/mm <sup>2</sup> $\pm$ standard error	Number of counts	Per cent germination $\pm$ standard error	Number of counts
35.8 $\pm$ 5.8	10	97.5 $\pm$ 0.2	4
94.2 $\pm$ 20.0	6	81.0 $\pm$ 2.6	3
132.5 $\pm$ 10.8	10	81.6 $\pm$ 2.5	5
152.5 $\pm$ 11.8	10	76.4 $\pm$ 2.4	5
194.2 $\pm$ 40.0	4	47.0 $\pm$ 2.9	6
266.7 $\pm$ 21.6	13	29.8 $\pm$ 2.4	8
316.7 $\pm$ 24.2	8	36.4 $\pm$ 1.3	9
438.5 $\pm$ 17.5	7	21.6 $\pm$ 1.5	5

**The effect of nutrient vs. non-nutrient agar:** In these experiments dilute spore suspensions were employed and the same suspension was dispensed on standard nutrient agar (with peptone and dextrose—see *methods*) and non-nutrient agar. The results on the non-nutrient agar were highly variable although in each of the 5 experiments run, the per cent germination on the non-nutrient agar was significantly lower than on the nutrient agar (Fig. 3).

A few experiments with and without phosphate buffer (on non-nutrient agar) were carried out in order to test Potts' (1902) suggestion that phosphate limits germination. The results showed no difference between the two conditions.

It should also be noted here that, in keeping with the above observations,



spore germination on washed agar is especially poor, although no quantitative studies were made of this phenomenon.

**The effect of the age of the spores:** Since it is a well established fact that in many species the spores must age a sufficient length of time for optional germination, spores varying from 2 to 15 days were compared and it was found that under similar environmental conditions their germination capacity was identical. Presumably in very old spores the per cent germination would steadily decline but no tests were made on this point.

**Discussion.** A reduction of germination in dense spore populations is not an unusual situation and is prevalent in many fungi (see Cochrane, 1958). Sometimes the concentration affects the rate of germination rather than the final per cent germination, as Ryan (1948) showed for *Neurospora crassa*; but this is consistent with the idea that more than one mechanism might be involved for different species. In some cases the results might be interpreted in terms of inhibitory substances given off by the spores themselves, and in others it might be competition for a key substance, such as Doran's (1922) evidence of the limiting effect of oxygen.

The selective advantages of such a phenomenon is obvious. In the first place it prevents germination in the sorus and secondly prevents the unnecessary quick germination of all spores thereby spreading germination over a longer period, which is a greater insurance against sudden adverse environmental conditions. It is interesting to note here, that in the case of the true slime molds (Myxomycetes), Smart (1937) showed the opposite phenomenon and high spore concentrations increased the per cent germination. Of course in the Myxomycetes the spore products, either directly or after a few cell divisions, are gametes, so in this case it is obviously advantageous to germinate in large numbers so as to insure fertilization by the simultaneous appearance of the sex cells. In the case of the cellular slime molds, on the other hand, there is no compelling evidence as yet that these spore products are gametes, and it is well known that a solitary spore will fare as well as a mass of spores. (This argument is included here not as evidence against sexuality in the Acrasiales, which is still an open matter, but merely as an hypothesis to account for the difference in germination activities between the two types of slime molds. Also it should be mentioned that Kerr and Sussman (1958) have shown that in *Didyium mgripes*, one of the Myxomycetes, it is possible to obtain the complete life cycle from one spore. The products of the one spore produce gametes which fuse. Therefore it must be assumed that the advantage of stimulation of germination in dense spore populations serves to aid cross fertilization between individuals).

In conclusion it should be emphasized that this study is fragmentary and there are many aspects of germination in the cellular slime molds that

deserve further investigation. Besides the point already mentioned, of whether or not the reduction of germination in dense populations represents an inhibition or a competition for a limiting substance, there need to be studies of the effects of temperature, humidity and light. Also, it is important to remember that no attempt has been made to examine the effect of the bacterial food supply; all the experiments reported here are in the absence of bacteria, for it was impossible with our methods to score the results in the presence of bacteria. Finally we have used only one strain of the species, and a variety of cellular slime molds should be tested.

#### SUMMARY

The ideal conditions for spore germination in *Dictyostelium mucoroides* at 22° C in the dark are those in which there is maximum humidity and a dilute spore suspension on a nutrient agar. Germination would begin after 1½ hours, reach 50 percent germination at about 2½ hours, and level off at about 98 per cent germination after 4 hours.

If under these conditions the spore density is increased, the initial rate of germination remains the same, but the final per cent germination after 5-6 hours is reduced.

Using dilute spore concentrations, it was found that non-nutrient agar gave a consistently lower final germination than nutrient agar.

No effect of the age of the spores (2-15 days) on their capacity to germinate could be detected.

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## AGGREGATION TERRITORIES IN THE CELLULAR SLIME MOLDS <sup>1</sup>

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The size of the area or territory which encompasses the amoebae that enter into an aggregate of a cellular slime mold is related to the process of initiation of aggregation as well as the size of the sorocarp. Each territory represents one aggregation and therefore one initiation event, and the size of any one fruiting body, or sorocarp, is primarily determined by the number of amoebae that enter an aggregation center. It is true that some of the cells may divide after the beginning of aggregation (Wilson, 1952; Bonner, 1960) but since all intake of food ceases some time before aggregation, there is no increase in bulk during the morphogenetic phases of the life-cycle, that is, while the aggregated cell mass progressively becomes transformed into a fruiting body.

It will be shown that regardless of the density of the amoebae, the territory size, under controlled environmental conditions, remains constant for any one species. This means that sorocarp size is largely controlled by cell density and that the problem of initiation is identical with the problem of the establishment of these rigid aggregation territories.

### MATERIALS AND METHODS

A simple method was used for the control of the amoeba density within a culture by controlling, in turn, the amount of bacterial food supply. Two per cent Bacto agar containing 6.2  $\mu\text{g.}/\text{ml.}$  of dihydrostreptomycin sulphate (Lilly) was poured into plastic Petri dishes which were marked on the bottom surface with squares of .102  $\text{cm}^2$ . A few loopfuls of *Escherichia coli* taken from a stock culture (grown on 1% dextrose, 1% peptone, 2% Bacto agar) were placed in 2 to 7 ml. of sterile distilled water. The density of the suspension was then determined in a Bausch & Lomb "Spectrometer 20" at 550  $\text{m}\mu$ . Appropriate dilutions were made to achieve a particular density and .09 ml. of this final suspension was evenly spread (with the help of an electric turntable and a sterile, bent glass rod) on the surface of one of the streptomycin agar Petri plates. In this way it was possible to obtain a range in food supply on the plates from approximately 500,000 bacterial cells/ $\text{mm}^2$  (= an optical density of 6.0) to 5000 bacterial cells/ $\text{mm}^2$  (= an optical density of 0.1). The spores of the slime mold were inoculated in three marked spots on each plate with a very fine, glass needle with a tip rounded in a small glass bead about .5 mm. in diameter, and consequently the inoculation points were confined to small, limited areas. Except for special

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experiments involving controlled temperatures and light, the dishes were incubated on a table in the center of the laboratory ( $25 \pm 3^\circ \text{C.}$ ). This was done after determining that the results were identical with those done at constant  $24^\circ \text{C.}$  conditions in continuous light, and in 12 hours of light alternating with 12 hours of darkness. Also in order to check the possibility that the streptomycin might be affecting the results in some way, some controls were run without the streptomycin, and no difference could be observed in the morphology or the size of the aggregation territories.

The counts of fruiting body density were made by inverting the Petri dish under a dissecting microscope and counting the number of fruiting bodies in 5 separate .102-cm.<sup>2</sup> areas and averaging the results. The mean aggregation territory was calculated directly from the sorocarp density. The radius of the aggregation territory was determined by considering each territory to be a circle. The size of fruiting bodies was determined two ways: in the case of very small ones the number of spore and stalk cells was counted directly, and in larger ones a camera lucida drawing was made of the stalk so that stalk lengths could be accurately determined.

## RESULTS

### *Sorocarp size*

According to the original description of the various cellular slime molds, each has its characteristic size. These sizes are usually indicated as stalk lengths, and under normal culture conditions this is an appropriate measure, although it is possible, by increasing the humidity of the air and decreasing the solute concentration of the agar, to obtain stalks of great length (Bonner and Shaw, 1957). For instance, in *Dictyostelium mucoroides* the largest are described as having stalks of 10 to 50 mm. or more (Raper, 1951) while under the specially humid conditions we obtained stalks up to 220 mm. in length. But this involved no increase in cell number; it is merely that the humid conditions favor prolonged migration and the transfer of the majority of amoebae into stalk cells, leaving very few spores. We are not concerned here with this aspect of size, but rather with size changes which reflect changes in cell number (or in dry weight).

As Raper (1951) has stressed, *D. mucoroides* may best be described as a complex, rather than a species, for the variability among strains is great, size being one of the significant variables. At one extreme there are the large forms typified by *D. giganteum* of Singh (1947) and at the other there is *D. minutum* of Raper (1941). The range in stalk height (under conditions which reflect cell number) is from .5 to 50 mm.

In the case of *D. discoideum* all the naturally occurring strains described thus far are all roughly the same size (a stalk height of 1 to 3 mm.) although Sussman (1955) has produced a mutant ("fruity") by U.V. radiation that is considerably smaller.

The genus *Polysphondylium* contains two species which differ significantly in size. The larger *P. violaceum* has a stalk length of about 5 to 30 mm. (Olive, 1902) while *P. pallidum* is roughly half the size.

*D. lacteum*, which has round rather than elliptical spores, is also a small species (.5 to 1.5 mm.). *Acytostelium leptosomum* (Raper and Quinlan, 1958), another

round-spored form, is small (1 to 1.8 mm.) but presents rather a special case in that the stalk is non-cellular and consists of a simple delicate cylinder of cellulose. The ultimate in small-size in the cellular slime molds is *Protostelium* (Olive and Stoianovitch, 1960) which consists of one cell which builds its own stalk (.07 mm. in height).

In all these cases the stalk length reflects size under optimum culture conditions. In the case of the larger forms this is a rich medium which produces a profusion of bacterial food supply. It is a well known and every-day observation that if a medium is used which supports poor bacterial growth, these large species will produce smaller sorocarps. Arndt (1937), in fact, made a series of observations

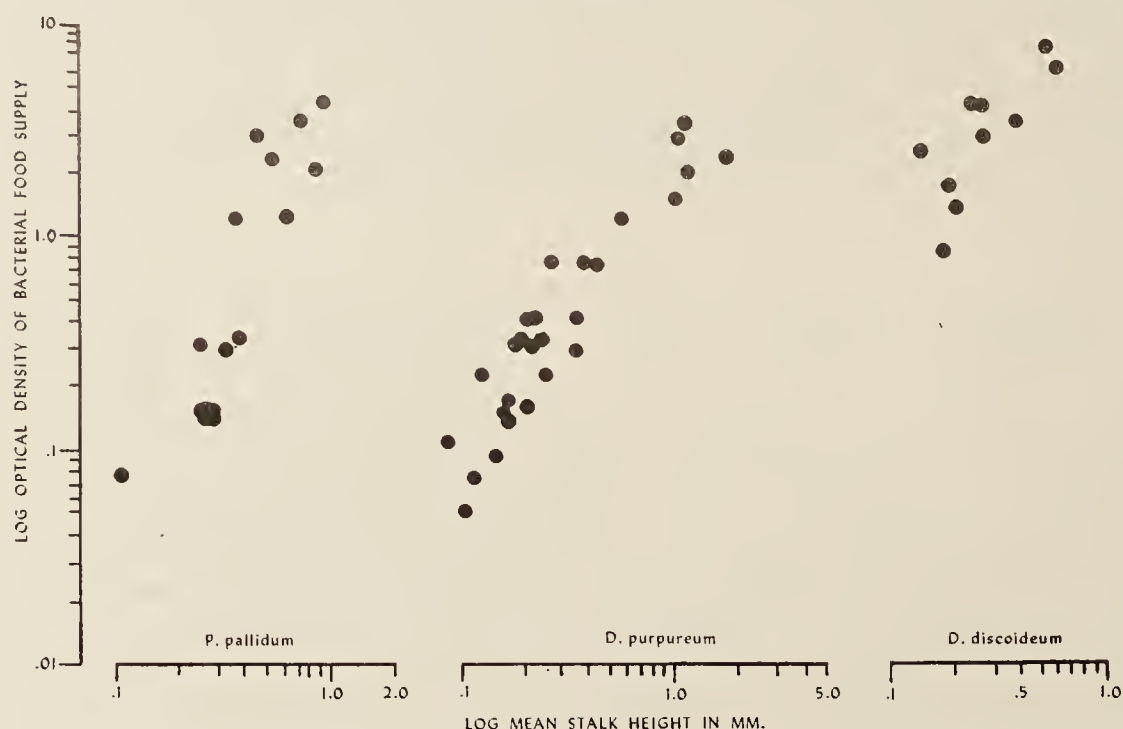


FIGURE 1. A graph showing the density of the bacterial food supply plotted against the mean stalk height which is used as an index of sorocarp size. Each point is an average of 20 stalks.

of cultures with different densities of bacterial food supply, and came to the conclusion that the size of the fruiting body was directly dependent upon amoeba density. As will be seen presently, this study is partly a quantitative exploitation of this commonplace observation.

Another frequent observation, repeatedly emphasized by Raper (*e.g.* 1951), is that the smaller species will not grow in rich media, but require dilute media. This is true for all the small forms mentioned above (*D. minutum*, *P. pallidum*, *D. lacteum*, *A. leptosomum*). In other words, for these species, the maximum size seems to be determined by the density of the bacterial food supply; there is an upper limit in the amount of food, above which all development is inhibited, possibly because of the production of inhibitory substances by the abundant bacteria.

With this in mind, different species were grown on different known concentra-

tions of *E. coli* on streptomycin agar. As can be seen from Figure 1, the size of the sorocarps increases with the increase in bacteria. The range indicated here only shows the lower limit of the species tested, but fails to show the upper limit.

Note that for the three species shown in Figure 1, each has a different minimum threshold. Some studies were also made of *D. minutum* which, as just mentioned, has a low maximum threshold (*i.e.*, does not develop with a rich food supply, probably because excessive bacterial growth produces inhibitory substances). It was a surprise to discover that it had a high minimum threshold necessary for aggregation. In other words it is a form which can only develop in a very restricted range of food densities. Other forms, such as *D. purpureum*, are much less particular and

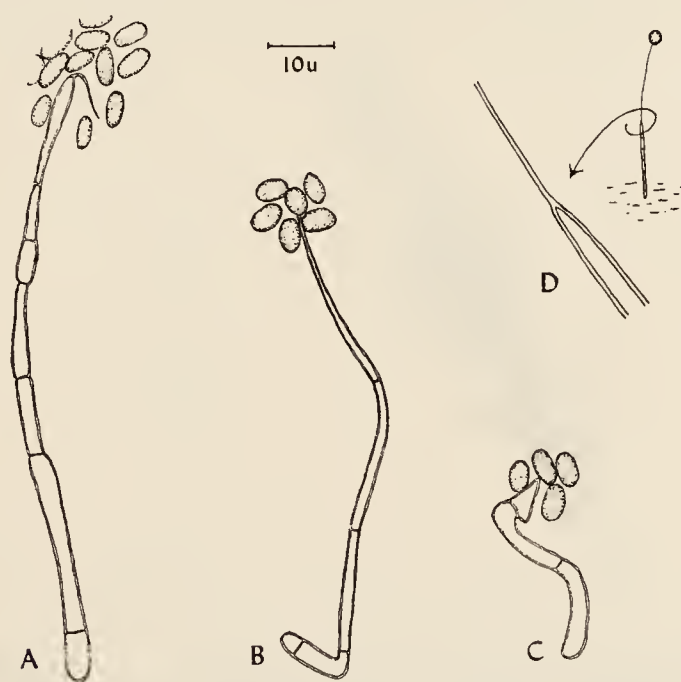


FIGURE 2. Camera lucida drawings of small sorocarps. A. *D. purpureum* with 7 stalk cells and 45 spores (not shown). Note the wisp at the end of the stalk. B. *P. pallidum* with 5 stalk cells and 6 spores. C. The smallest sorocarp obtained. It is *P. pallidum* with 3 stalk cells and 4 spores. D. *D. lacteum* showing a small stalk which midway becomes acellular.

can adapt to a wide range of concentrations, and can produce, as a result, a wide range of size in their fruiting bodies from ones much smaller than *D. minutum* to ones very much larger. This leads to an important point as far as *D. minutum* is concerned: one of the reasons for its small size is the fact that it normally develops in a restricted range of food density and because of the low value of this particular range, the mean sorocarp size is correspondingly small. There are undoubtedly other factors which also may limit size in *D. minutum*, a matter which is under further investigation.

In general, on any one culture dish, at any one bacterial density, the sorocarps were remarkably uniform in size. At the minimum thresholds for each species it was of interest to examine the morphology of the smallest sorocarps for any



possible effects caused by the size reduction. In some species the stalk cells appeared somewhat coarse and club-like (*D. mucoroides*, *D. purpureum*, *D. discoideum*) while in others the cells were beautifully tapered (*P. pallidum*, *P. violaceum*, *D. lacteum*) (Fig. 2). In the case of *D. lacteum* the smaller sorocarps had an acellular tip, giving them, at least at their anterior end, an appearance almost exactly resembling *Acytostelium*. This is not true of the larger sorocarps of *D.*

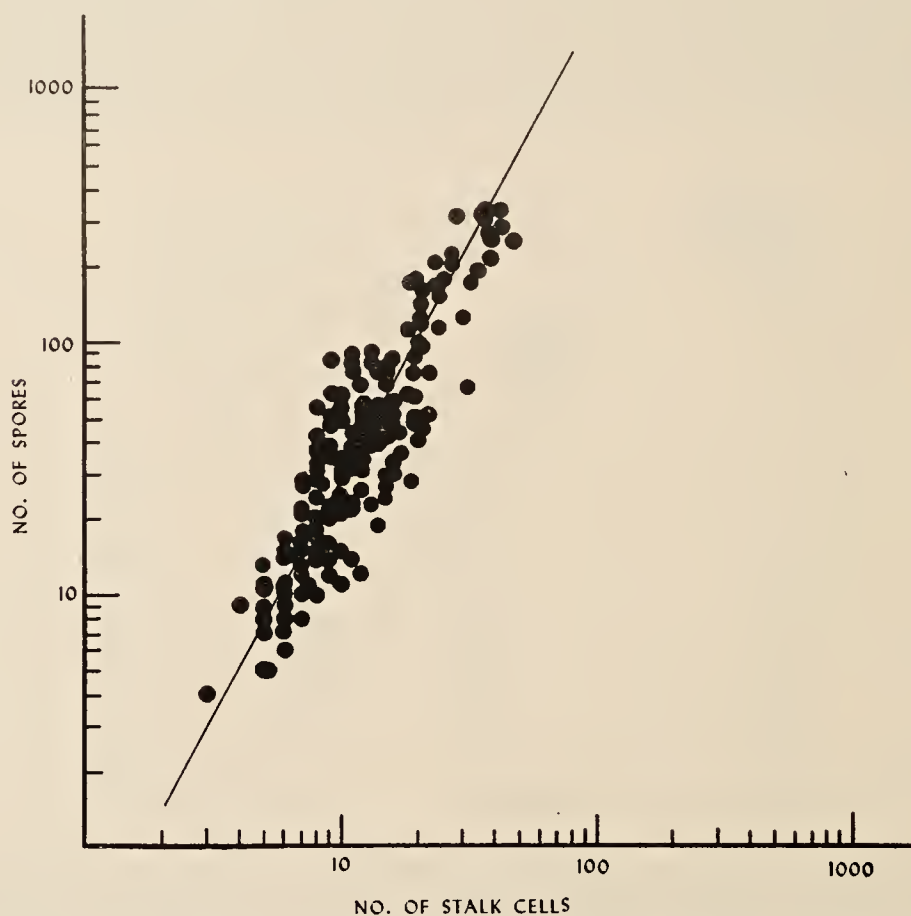


FIGURE 3. The log of the number of spores is plotted against the log of the number of stalk cells. These points are a composite of *D. purpureum*, *D. mucoroides*, and *P. pallidum*, as they showed no discernible difference among them.

*lacteum*. Occasionally in *D. purpureum* it was possible to observe sharp acellular wisps of stalk material at the anterior end of the stalk. The smallest fruiting body encountered in these studies of all the species examined was a seven-cell sorocarp of *D. purpureum* (three stalk cells, four spores).

Since it was possible to count the cells in small fruiting bodies, a study was made of the proportions of stalk to spore cells, this method having many advantages over the less direct ones previously devised (Bonner and Slifkin, 1949; Bonner, 1957). A number of counts were made for *D. mucoroides*, *D. purpureum* and *P. pallidum* (these latter ones were unbranched because of their small size, but as they showed no significant difference among them, they have all been plotted

together (Fig. 3). The character of the allometric relation is identical to the one previously described for larger pseudoplasmodia (Bonner, 1957).

### *Aggregation territories*

While size and food supply (which is a reflection of amoeba density) are directly proportionate, fruiting body density is independent of size. That is, regardless of the density of amoebae, the size of an aggregation territory remains constant (Fig. 4). It is of interest to note that Arndt (1937) came to a similar conclusion, although he made no quantitative determinations to support his observations. To emphasize the point, it is space, not the number of cells, that is important in determining the size of an aggregation territory. If there are 100 cells in a unit

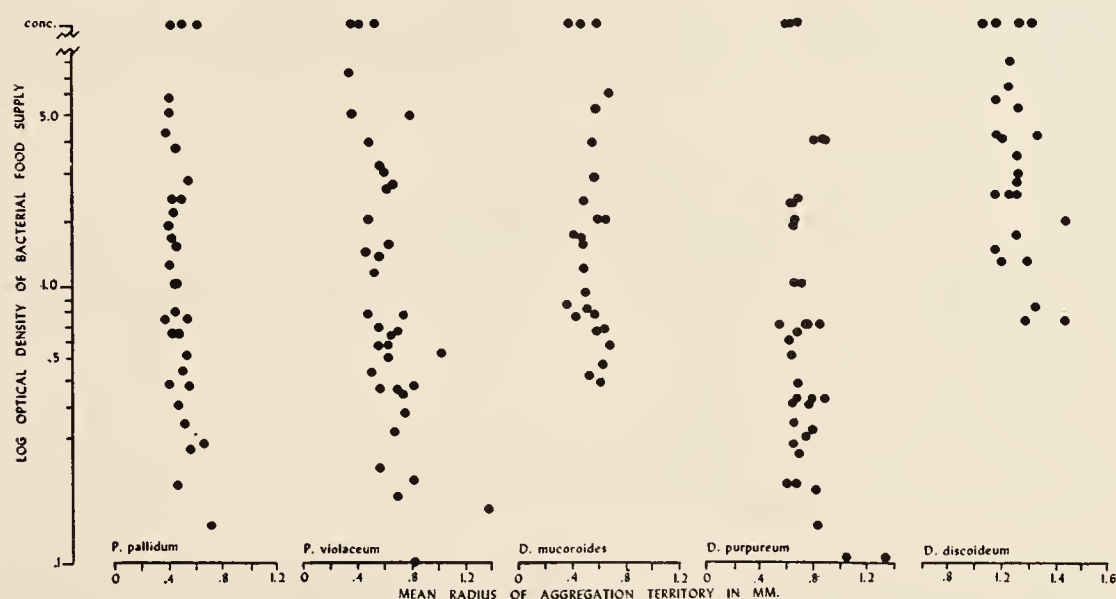


FIGURE 4. Five graphs for different species, showing the 'radius of the aggregation territory under conditions of different bacterial food densities. The top points labeled *conc.* are on 2% agar without streptomycin and a heavy layer of bacterial paste smeared evenly over the surface. Each point is an average of 5 squares (.102 cm.<sup>2</sup>) on one culture dish.

space, they will produce one fruiting body of 100 cells; if there are 1000 cells then they will produce one fruiting body of 1000 cells. Of course this statement is oversimplified as it ignores the possibility of cell division and the fact that not all the cells may enter the aggregate but some are left behind (and in fact their number can be accurately determined).

Some experiments were also run at higher concentrations of bacteria to determine if the constancy of the size of the aggregation territory was affected by very high amoeba densities. This was first done on non-nutrient plates covered with a heavy layer of bacterial paste for all five species, and as can be seen in Figure 4 results compare with the more dilute plates. A further experiment was run on *D. purpureum* using full nutrient agar (10 gm. peptone, 10 gm. dextrose, .96 gm. Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 1.45 gm. KH<sub>2</sub>PO<sub>4</sub>, 1000 ml. H<sub>2</sub>O, 20 gm. agar) and the same diluted in half and by one fourth (with the exception of the agar content

which remained at 2%). The results showed no obvious difference between the three concentrations of nutrients, and the range of twelve runs extended from a territory radius of .41 to .56 mm. which overlaps, but is significantly lower than the range using non-nutrient agar. This discrepancy is unexplained although there are a number of possibilities, such as the specific effects of the nutrients themselves on territory size, a possibility which will come up again in discussing the environmental factors which affect the aggregation territory.

As a check to determine if the same result could be obtained by using radically different techniques, two further experiments should be mentioned. In one the amoebae of *D. discoideum* were centrifuged free of bacteria and suspended in a salt solution (see Bonner, 1947) in van Tieghem cells with one end sealed with a coverslip. A microscope slide was then sealed to the other end of the cell, and the whole preparation inverted after the amoebae had settled on the coverslip. They were thus attached to glass and retained in small moist chambers which were incubated at 20° C. in the dark (except for hourly examinations under the micro-

TABLE I  
*An experiment on territory size using D. discoideum on coverslips in small moist chambers (van Tieghem cells)*

Amoeba density: amoebae/mm <sup>2</sup> .	Mean sorocarp height in mm.	Total number of sorocarps per coverslip	Mean radius of the aggre- gation territory in mm.
246	.76	26	1.32
318	.77	23	1.41
618	1.08	22	1.44
1040	1.05	28	1.28
2570	.86	23	1.41

scope). At very high and very low amoeba concentrations the fruiting bodies were few and small, but in a large range of intermediate concentrations the territory size remained constant, while the mean stalk length tended to increase, as would be expected from previous results (Table I).

In the other experiment the liquid culture technique of Gerisch (1960) was employed and after the growth phase the amoebae of *D. purpureum* were harvested, plated out in different concentrations on the streptomycin agar plates and incubated in room conditions. This method has the advantage of making it possible to determine amoeba densities directly. If Figure 5 and Figure 4 are examined, it is obvious that the ranges of the territory size using the two techniques are comparable.

In order to examine the formation of aggregation territories in more detail, time lapse motion pictures were taken of the aggregation of *D. purpureum* at four different bacterial food densities on streptomycin agar. It was possible, in each of the cases, to determine the amoeba densities which were 54, 147, 174, and 356 amoebae/mm.<sup>2</sup>, respectively (included in Figure 5). The only significant difference that could be observed among the four cases was that the resulting fruiting bodies were progressively larger as the amoeba density increases. As far as the time of formation of the aggregates and the general blocking out of the territories, they were all similar. For instance in none of the cases was there any



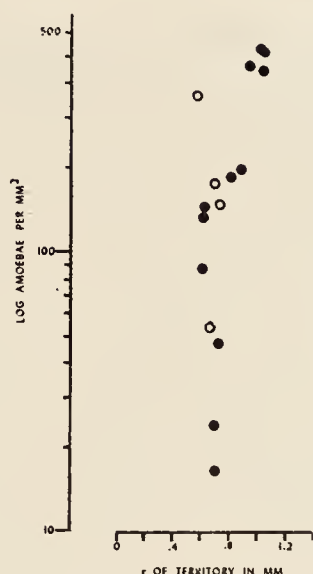


FIGURE 5. A graph showing the radius of the aggregation territory at different known amoeba densities for *D. purpureum*. The solid dots involve the Gerisch (1960) liquid culture technique and the hollow circles were taken from a motion picture of cultures prepared by the streptomycin agar technique described in Materials and Methods.

evidence of the formation of more numerous territories, some of which would then disappear. Each center, once formed, remained stable and there was no subsequent disintegration.

If the rate of appearance of new centers is plotted against time for each of the amoeba densities, it is clear that they do not appear steadily, but with considerable irregularity. However, under these conditions of constant illumination there was no evidence of bursts of aggregation such as Shaffer (1958) obtained with alternate darkness and light.

As is evident from Figure 4, each species has a characteristic territory size. They are listed in order of magnitude for the various species in Table II. A few determinations were also made of *D. minutum* and *D. lacteum*, which indicated that they are both on the lower end of the scale. This means that to a minor extent the small size of these two species and *P. pallidum* might be accounted for by their small territory size.

The fact that environmental conditions affect the density and size of fruiting bodies has been appreciated for a long time. Potts (1902) and Harper (1932) showed that light produced more numerous and smaller fruiting bodies. This was confirmed by Raper (1940), who also showed that a slight decrease in humidity

TABLE II

*The average radius of the aggregation territories of different species*

Species	No. of cases	Mean radius in mm.
<i>D. discoideum</i>	25	1.27
<i>D. purpureum</i>	36	.73
<i>P. violaceum</i>	39	.63
<i>D. mucoroides</i>	24	.53
<i>P. pallidum</i>	31	.49

would elicit the same effect. Bradley, Sussman and Ennis (1956) studied the influence of various chemical agents upon aggregation and they found that histidine in suitable concentrations produced more numerous smaller fruiting bodies, while adenine had the reverse effect. The effect of histidine has been confirmed by recent studies of Krichevsky and Wright (personal communication).

Using the test system described in this paper and *D. purpureum* as the test organism, Heller and Miles (1961) have shown that light is exceedingly effective and that as the intensity increases the aggregation territory becomes correspondingly reduced. They also showed that humidity, in the dark, had a very small effect as compared to the light effect, and that while the aggregation territory became reduced in size with decreasing humidity it soon reached a minimum (very roughly in the neighborhood of 98% relative humidity) and then increased rapidly as the relative humidity of the surrounding atmosphere approached 95%.

In a parallel study Opderbeck (1961) examined the effect of histidine and also successfully repeated the results of the previous workers in the dark, but in the light he found that the histidine had the reverse effect; that is, it produced, in all the concentrations tried (from  $10^{-1}$  to  $10^{-3}$  M), an increase in the size of the aggregation territories as compared to the controls. It is hoped that ultimately further study of these effects (which is in progress) may shed some light on the factors which are responsible for the delineation of the aggregation territory.

#### DISCUSSION

At the moment the mechanism of territory formation is unknown. It is, of course, possible to suggest many hypotheses, two of which will be mentioned here.

(1) Any cell could become so altered in its state that it would produce an inhibiting substance which diffuses outward and prevents any other cell, within a given radius, from achieving the same state. Clearly the distance the substance diffuses would be independent of the number of cells within a territory. One must also presume that the cell producing the inhibitor lies at the center of the future aggregation pattern, similar to Shaffer's (1961) "founder cell" in *P. violaceum*.

(2) The above hypothesis assumes two separate functions for the keystone cell: inhibition and the subsequent initiation of acrasin production. It might be possible to explain the whole phenomenon solely on the basis of acrasin diffusion. If we assume that the original puffs of acrasin are not carried outward from cell to cell by the Shaffer (1957) relay system, but diffuse from one cell or a small group of cells, and if we assume that a certain concentration of acrasin prevents other cells from becoming acrasin-emitting pace-makers, then this original diffusion gradient of acrasin can be unaffected by the number of cells in a territory and therefore delineate the aggregation territory. Unfortunately, these hypotheses and any other we might invent have far too many assumptions and are in urgent need of testing by experiment.

In relating this work to those of others it first should be mentioned that although Sussman and Noël (1952) and Sussman and Sussman (1961) have made a study of the relation of amoeba density to the number of fruiting bodies, they did not measure either territory size (fruiting body density) or sorocarp size, and therefore it is impossible to compare their work with the present study.

On the other hand, the aggregation territories are obviously related to the problem of the initiation of aggregation and it is pertinent to examine the "initiator cell" hypothesis of Sussman and his group (*e.g.* Sussman and Ennis, 1959; Ennis and Sussman, 1958). This hypothesis assumes that special "initiator cells" are in a fixed proportion to the total cell number: in *D. discoideum* one cell in every 2200 is presumed to be an "initiator cell," while in *D. purpureum* it is one cell in every 300. We have made some cell counts for these two species under threshold conditions where the amoeba density is just sufficient to produce aggregation and fruiting. If the total number of cells per territory is determined (*i.e.*, the number of cells for the sorocarp as well as the number of cells that failed to enter the aggregate) they average 1032 for *D. discoideum* and 150 for *D. purpureum*. In other words there are roughly twice as many centers as there are "initiator cells." However, there are so many other ways of showing that aggregation occurs in small populations of cells below the number predicted from the "initiator cell" hypothesis that the hypothesis may no longer be considered tenable (Bonner, 1960; Konijn and Raper, 1961; Gerisch, 1961).

But perhaps the far more important point is that contrary to the "initiator cell" hypothesis, aggregation is not determined by a cell which holds a strict proportion to the other cells in a population; it is completely independent of the other cells (provided a sufficient cell density is maintained). The only factor which clearly and absolutely controls the initiation process is space: the aggregation territory is, for each species under given environmental conditions, a fixed entity.

These conclusions are entirely in keeping with those of Shaffer (1961), who has shown that existing aggregations in *P. violaceum* are capable of inhibiting the further production of founder cells, even in populations of cells that are not entering streams. According to Samuel (1961) the earliest manifestation of the establishment of the aggregation territory (*i.e.*, initiation) is a regional depression in the rate of cell movement. This is followed, as Shaffer (1961) has shown, by the appearance of a cloud, an area of relatively dense amoebae. In *D. mucoroides* and *D. purpureum* a true aggregation center, with the eventual appearance of incoming streams of amoebae, is only evident after these two initial stages. To completely understand the factors which control initiation and the distribution of the aggregation territory, it will be necessary to provide an explanation for all the events that lead up to the aggregation process itself.

One final point that may have some bearing on future experiments: in previous studies (Bonner, 1960) it was shown that if a small group of cells is isolated by scraping away the cells all around, they often dispersed or disintegrated after aggregation, and in *D. purpureum* the aggregates showed a tendency to produce abnormal sorocarps. In the present study sorocarps of comparable size never disintegrated or showed signs of abnormality. The reasons for this difference are not known but they raise the interesting possibility that if an aggregation territory is isolated and not surrounded by other aggregation patterns, it lacks certain peripheral chemical influences, which results in disintegration or abnormality. The question really is whether or not the cells in neighboring territories remain in communication one with another during aggregation and the later states of morphogenesis.



## SUMMARY AND CONCLUSIONS

1. The area of the aggregation territory in the cellular slime molds is constant at different cell densities and therefore the number of amoebae that aggregate in any one territory varies directly with the cell density. As a result sorocarp size in the cellular slime molds is a function of the density of the amoebae prior to aggregation.

2. The mechanism whereby the territory size is determined is not known, although clearly the problem of the initiation of aggregation is related to the establishing of fixed territories. Since their establishment is independent of cell number we may propose the hypothesis that initiation is determined solely by space or distance.

3. There are a number of conditions which frame these general conclusions. The territory size is characteristic for each species and is constant only under a particular set of environmental conditions. Also the relation obviously only applies when the amoeba density is sufficient for aggregation, and each species has a specific range of densities which permit aggregation and fruiting.

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## Evidence for Gas-Induced Orientation in the Cellular Slime Molds<sup>1</sup>

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### INTRODUCTION

In many lower organisms which send stalks into the air, it is well known that commonly the stalks orient at right angles to the substratum. This is true for numerous small filamentous fungi, myxomycetes, cellular slime molds, and myxobacteria. In these cases the minute stalks appear to be relatively or completely insensitive to gravity, and if they are photo- or thermotropic, the right-angle orientation can be demonstrated only in the absence of temperature gradients and with uniform illumination (or in total darkness).

In the cellular slime molds there is an aggregation of uninucleate amoebae, and the resulting cell mass may crawl about for a period as a unit, finally righting itself and rising into the air. As it rises or culminates, it forms a delicately tapered stalk which supports a rounded group of amoebae, each one of which turns into a spore in the final apical sorus.

Evidence will be presented in this paper to support the hypothesis that the orientation of this culminating cell mass is achieved by its giving off a volatile substance, a gas, which has a strong orienting effect on the pseudoplasmodium that produces it. The culminating cell mass orients in a diffusion gradient of the gas away from the regions of high concentration (negative chemotaxis). In this way a single fruiting body which is rising from an agar surface and is constantly emitting the gas will automatically orient at right angles from the substratum. Although not considered in this paper, there is considerable

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evidence that orientation in a number of different filamentous fungi may be interpreted by a similar hypothesis. We would, therefore, want to suggest tentatively that gas orientation is a general phenomenon among small, stalked, lower organisms.

The first evidence for gas orientation in fungi comes from the early work of Burgeff (1924) on volatile sexual substances in the mating reactions of mucors. However, until very recently, this work has met with no more than varying degrees of skepticism. We are indebted to Banbury (1955) for the first really convincing demonstration that heterothallic mucors do give off volatile substances and that they are responsible for the orientation of opposite-sexed hyphae toward each other and for the orientation of hyphae of the same sex away from each other.

The first inkling as to the mechanism of right-angle orientation in cellular slime molds came from an observation of two senior students, J. H. Rorke and G. Rosenthal, working in this laboratory in the spring of 1959. We knew (an example had been recorded on a time-lapse film in 1941) that if a migrating slug of *Dictyostelium discoideum* was cut into three portions and if the pieces kept their relative positions, then the anterior end would lean forward as it culminated, the posterior portion backward, and the middle portion somewhere in between (Fig. 1). There was no known mechanism for this orientation, and Rorke and Rosenthal (1959) set out to determine whether perhaps, the front portion of the slug contained faster cells than the hind portion and the anterior and posterior orientation reflected these different rates of movement. In systematic tests they noted in one case that before culmination the anterior piece migrated and by chance stopped and culminated between the posterior and the middle piece. Nevertheless, the orientation was the same as before (e.g., Fig. 1); in other words the middle piece now leaned forward. By moving the pieces with a hair loop they proceeded to try all the different permutations of the order of the three pieces (as in the shell game). In all cases, without regard to their origin, the outside cell masses culminated away from the group. In other words, their orientation appeared to be the result of something their neighbors were actively giving off, and they were repelling one another by this means. Let us now examine in detail the evidence to support the hypothesis that this active agent is a free diffusing gas.

A few general points about materials and methods should be men-

tioned, although most of this information will appear in the appropriate sections. *Polysphondylium pallidum* was used as the principal organism for this study as it produced consistent and delicately tapered fruiting bodies that were found to be especially suitable for observation and recording. However, all the major points established for this species were also tested on *Dictyostelium discoideum*, *D. mucoroides*, and *D. purpureum*; it is felt, therefore, that the phenomena described are general for the cellular slime molds.

A number of the measurements on orientation are in terms of angle of deviation from the perpendicular (i.e., at right angles to the substratum). These are determined directly from camera lucida drawings of mature fruiting bodies where a straight line is drawn from the base of the stalk to its apex as it disappears into the sorus, and the deviation of this angle from 90 degrees is measured with a protractor.

#### A DESCRIPTION OF ORIENTATION CAPABILITIES

*Two fruiting bodies repelling each other.* *Polysphondylium pallidum* was grown on nonnutrient agar with *Escherichia coli* spread thinly over the surface. At the end of aggregation two centers close to one another were isolated with a hair loop by scraping the surrounding area clear of all cells. The whole block was cut out with a knife and placed in a plastic (Lucite) rectangular observation box ( $7.5 \times 3 \times 2$  cm). This in turn was put in the dark at  $23 \pm 1^\circ$  C. After culmination the two fruiting bodies were drawn with a camera lucida.

In the first series of 36 cases the distance between the bases was measured, and whether or not the two fruiting bodies leaned away from one another was recorded. The range of distances between the bases was from 0 to 2.14 mm, and it was evident that repulsion occurred only between 0 and 0.80 mm; if the distance was greater than 0.80 mm, the fruiting bodies had no orienting effect upon each other. At 0 mm, when their bases touched, the angle of both fruiting bodies was almost exactly 45 degrees, and as the distance between the bases increased, the angles decreased.

Another series of experiments was then performed in which an attempt was made to have the distance between the two centers approximately 0.30 mm. In 17 cases the mean distance was 0.33 mm, the minimum being 0.17 mm and the maximum 0.50 mm. The angle of deviation from the perpendicular (right angles to the substratum) was

then recorded for each of the 34 fruiting bodies involved and found to be 18.1 degrees  $\pm$  a standard deviation of 13.2 degrees.

A control series of 55 single fruiting bodies were run under similar conditions; they produced a mean deviation from the perpendicular of 1.8 degrees with a standard deviation of  $\pm$  13.3 degrees. Using the *t* test, it can be shown that the experimentals are significantly different from the controls ( $t = 5.7$ ;  $P = < 0.001$ ).

*Fruiting bodies culminating near a right-angle corner.* These experiments were designed to determine the effect of culminating at the base of an agar cliff. Two methods (which produced identical results) were employed. In one, a single aggregation center of *P. pallidum* was isolated on an agar strip and a small rectangular block of agar was placed at right angles to it. In the other, the face of the cliff itself held the center so that culmination took place in a roughly horizontal plane. In either case there was a right-angle corner of agar with the center near the corner (Fig. 7). They were placed in the plastic rectangular observation chambers and put in the dark at  $23 \pm 1^\circ$  C.

In the whole series of 70 cases there was a range of distances from the corner of the blocks to the base of the fruiting body from 0.10 mm to 4.00 mm. It is clear that if the fruiting body is 0.09 mm or less from the corner it is repelled and leans away. If the distance is greater than 0.90 mm, the orientation is at right angles from the substratum.

It is possible to select all those cases below 0.50 mm (a range of 0.10–0.47 mm) which give a mean distance of 0.33 mm to the corner. If the angles of deviation from the perpendicular are now determined for each of these 38 cases they average 18.1 degrees  $\pm$  a standard deviation of 12.9 degrees. Note that this is almost identical to the mean distance and angle for the case of repulsion between two fruiting bodies, which is surprising for one would have expected the two fruiting bodies to show greater repulsion. The explanation might be that the two fruiting bodies, as they rise into the air, become separated by an even greater distance, because of the angle of rise. Also they probably do not rise precisely together, thereby further increasing the effective distance. Needless to say, these right-angle corner experiments also differ significantly from the 55 controls ( $t = 5.9$ ;  $P = < 0.001$ ).

*Glass rod experiment.* In order to obtain some indication of the degree of sensitivity of the orientation phenomenon, a thin glass rod was plunged into the agar close to a single aggregation center of *P.*



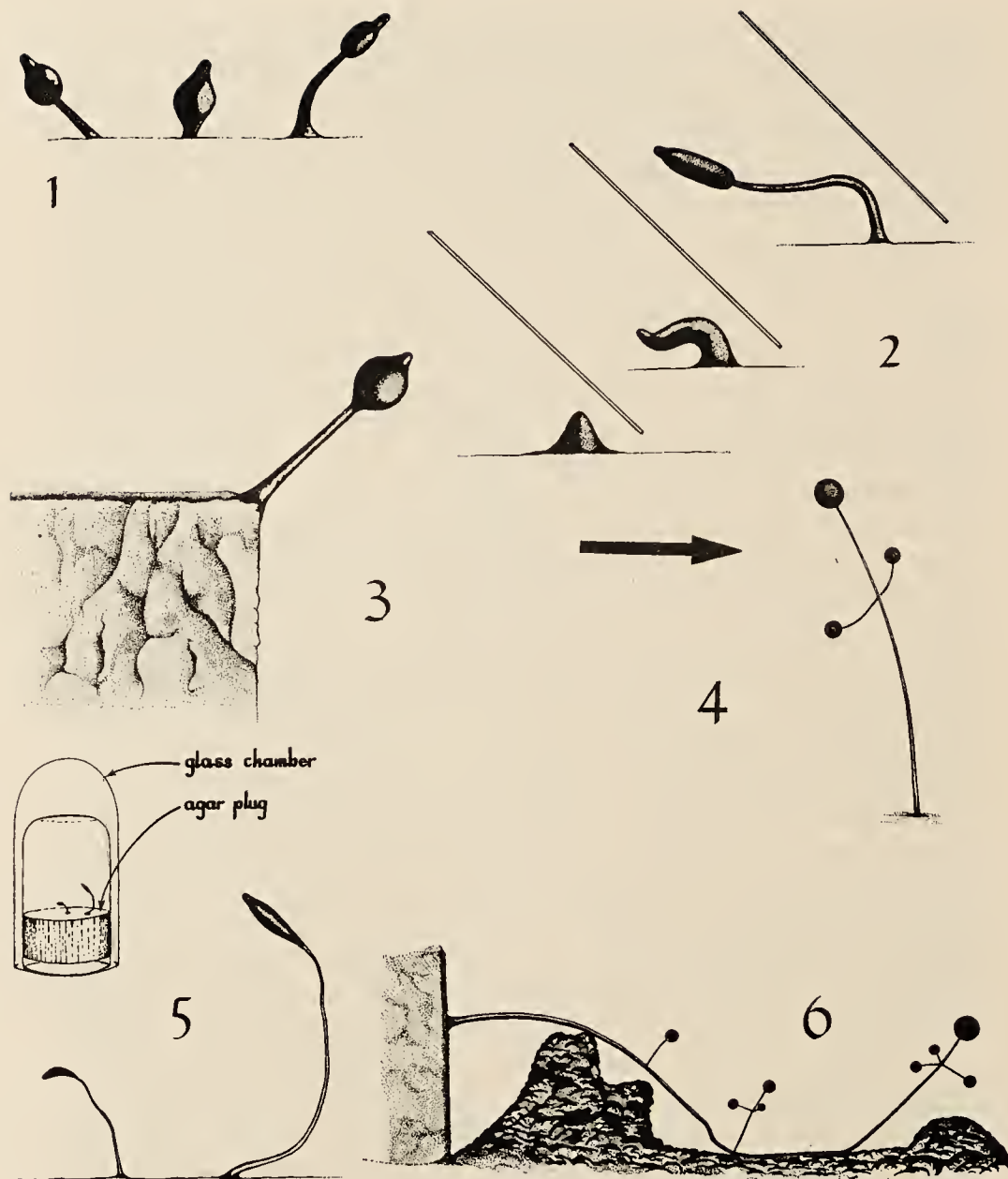


FIG. 1. A drawing from a film of one migrating pseudoplasmodium of *Dictyostelium discoideum* cut into three portions, each one fruiting separately (height of right-hand sorocarp is ca. 1 mm).

FIG. 2. Three successive stages of a sorocarp of *Polysphondylium pallidum* orienting under a coverslip held at an angle (height of upper sorocarp is ca. 0.5 mm).

*pallidum*. In all other respects these were handled in the same way as the above experiment.

The mean diameter of the glass rods was 0.11 mm (the range was from 0.06 to 0.19 mm), and the mean distance between the glass rod and the base of the fruiting body was 0.30 mm (the range was from 0.09 to 0.56 mm). In 32 cases the mean angle of deviation away from the rod was 7.3 degrees  $\pm$  a standard deviation of 11.5 degrees. This was found significantly different from the control ( $t = 1.95$ ;  $P = 0.06$ ). In other words, the fruiting bodies detected the presence of a rod 0.11 mm in diameter, 0.33 mm distant, and leaned in the opposite direction. However, the effect of the rod is less than that of a wall or another fruiting body at a comparable distance.

*Miscellaneous experiments in environments of different configurations.* When a solitary fruiting body is developing on a flat substratum it will orient so that the distance between its tip and the substratum is equal on all sides, thus producing the right-angle response. If the fruiting body rises from a right-angle corner, it will rise at 45 degrees, again equalizing the distances to the substratum. By holding a small piece of coverslip over the agar at various angles with a micromanipulator, it was possible to show that this equal distance is maintained even in small wedge-shaped spaces; the fruiting body emerged from the middle of the wedge (Fig. 2). If the coverslip was added after the cell mass had begun to rise, the rising sorocarp would alter its direction to accommodate to the new confining space, indicating that orientation is taking place continually during culmination.

Finally, it is a common observation to see a fruiting body emerge on the upper edge of a cut agar block. In this case, there will be a space of 270 degrees, for possible orientation. Invariably the fruiting body will jut out so that it is approximately 135 degrees from the block

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FIG. 3. A sorocarp of *D. discoideum* culminating at the upper edge of an agar block (height is *ca.* 0.5 mm).

FIG. 4. A sorocarp of *P. pallidum* that has fruited in the wind tunnel. The direction of the wind is indicated by the arrow (height is *ca.* 0.8 mm).

FIG. 5. Fruiting of *P. pallidum* in a small confined space (21 mm<sup>3</sup>). Note the lack of orientation in the camera lucida drawings of the two sorocarps (right-hand one is 1.1 mm high).

FIG. 6. A camera lucida drawing of *P. pallidum* fruiting near a layer of charcoal (the sorocarp is 1.2 mm long).



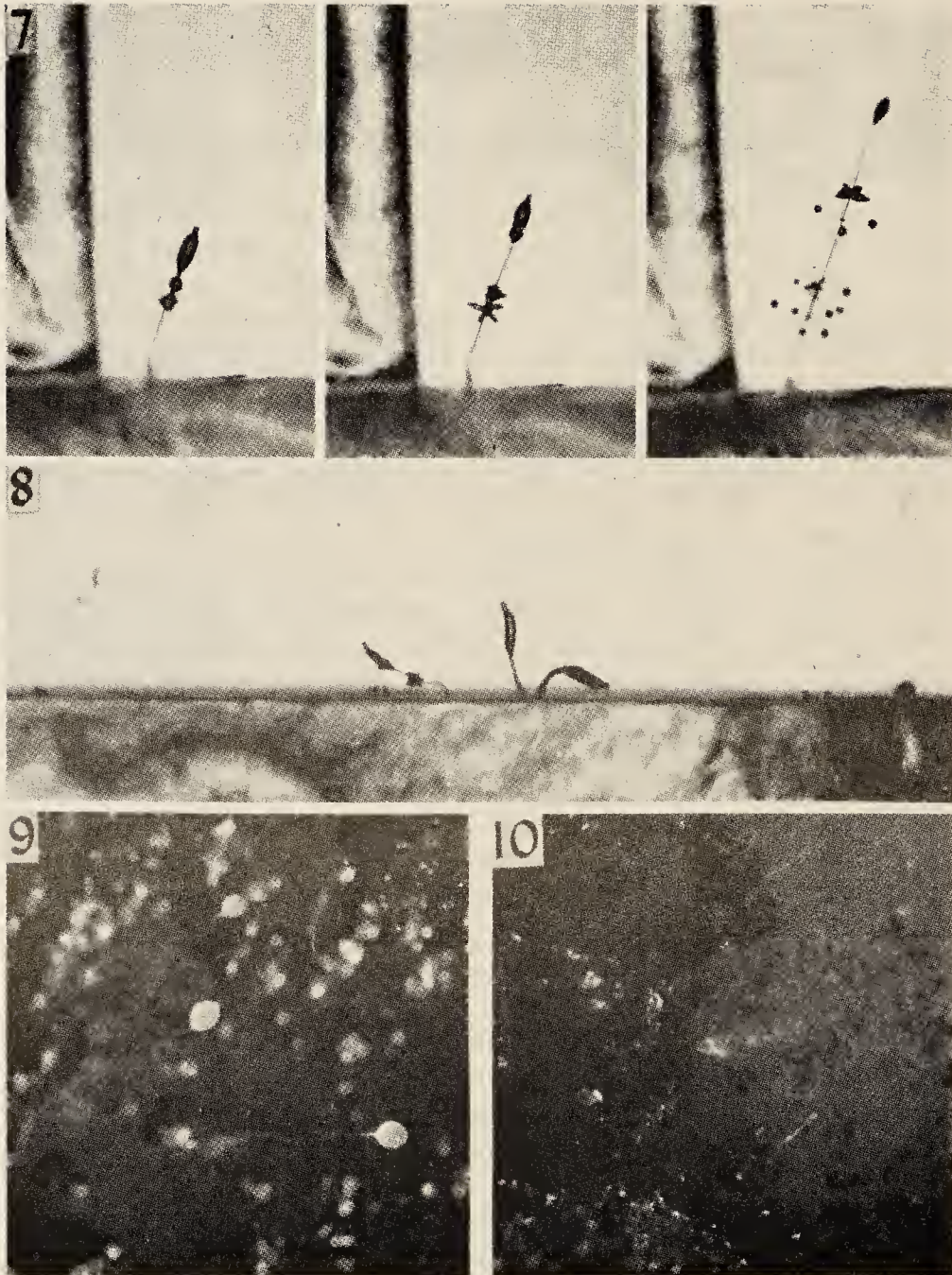


FIG. 7. Three successive photographs of a sorocarp of *Polysphondylium pallidum* rising near the corner of an agar cliff. Note that the fruiting body orients away from the agar corner.

FIG. 8. A photograph showing three sorocarps developing in mineral oil. Note the lack of orientation under these conditions.

FIGS. 9 and 10. Two surface views showing the orientation of sorocarps toward heaps of charcoal.



on each side (Fig. 3). Again it will seek a position that is equidistant from all solid objects in its environment.

#### EXPERIMENTS THAT RULE OUT VARIOUS PHYSICAL AGENTS THAT MIGHT BE RESPONSIBLE FOR THE ORIENTATION

*Heat.* It is well known that culminating fruiting bodies are extremely sensitive to light and temperature (just as it is known that they are insensitive to gravity). They will orient toward light and toward warmer regions (for a review see Bonner, 1962). This fact was frequently brought home to us in this study, for the slightest light leak in our dark boxes or the smallest of temperature gradients (i.e., an incubator 6 feet away from the dark boxes in a constant temperature room with air circulation) caused orientation and produced spurious results.

But in the case of fruiting bodies in the absence of an external light source or heat gradient, there remains a self-initiated mechanism by which the right-angle orientation is achieved. Is it possible that the rising sorocarp, which is actively metabolizing, might be a heat source that orients within its own heat gradient? The only difficulty with such a scheme is that the cell masses are known to go toward warmer regions and this would mean that two fruiting bodies would lean toward each other, which is precisely the opposite of the way they behave.

*Radiation.* If it is assumed that the cell mass produces some form of ray, then it must follow that this ray is effective at a distance of only 0.9 mm or less and that it can be reflected from the surface of agar. Various other substances have been tested; glass, iron, plastic (Lucite), aluminum, porous clay, and it is found that fruiting bodies tend to lean away from them as they do in the case of agar. It would seem unlikely that all these substances are equally able to reflect the hypothetical ray. Some of the experiments that will be described shortly also argue strongly against radiation, but since the case is such a weak one to begin with, there seems little point in pursuing it further.

*Electricity.* As was noted above, a great many different substances, including metals, all produce the same effect on the sorocarp as agar, and, therefore, it is presumed that surface changes could hardly be involved in orientation. However, Dr. James Gregg of the University of Florida pointed out to us that Parafilm "m" when held near mature fruiting bodies, strongly attracted them the way an electrified rubber rod will attract hair. If the fruiting bodies culminate near a piece of

Parafilm they tend to be highly distorted and abnormal. The peculiar convolutions result in the fruiting bodies being oriented in all different directions. While these experiments are not conclusive, they do not support an electrical hypothesis of orientation.

#### THE GAS ORIENTATION HYPOTHESIS: ATTEMPTS TO MODIFY AND INTERFERE WITH THE DIFFUSION PATTERN

Let us now examine a series of experiments which are all critical for the gas orientation hypothesis. It should be pointed out from the beginning that none of them will prove that this hypothesis is correct, but in each case the hypothesis is given strong support.

*Orientation in moving air.* An attempt was made to have the fruiting bodies culminate in a current of air. After various difficulties, Professor D. C. Hazen of the Department of Aeronautical Engineering was kind enough to come to our aid, and a small plastic "micro wind tunnel" was constructed in which the slime mold base would be flush with the inside chamber (which was  $3 \times 4$  mm). This was attached to an aquarium aeration pump and the air was passed through a stone bubbler in a flask of water to saturate the air before it entered the chamber. By altering a screw valve in the system it was possible to vary the air speed from about 30 to 200 cm/sec. It must be remembered that these are maximum speeds for the center of the lumen of the chamber, and that, because of friction and irregularities of the surface of the inside of the wind tunnel, there would be many regions of much lower velocities for any particular valve setting.

A series of tests were run at three different speeds: approximately 30, 100, and 200 cm/sec. There were no differences among these three groups, and in fact the main result can be easily stated: In air velocities of 30 to 200 cm/sec, the fruiting bodies leaned into the wind (Fig. 4). In 26 separate experiments there was only one case where more fruiting bodies pointed downwind than upwind (2 to 1). In fact, of all the 98 fruiting bodies recorded, 76 pointed upwind, 7 downwind, and 15 were vertical and showed no response. The only other instance of fruiting bodies pointing downwind was one in which there was a very large number of fruiting bodies (22 in all: 13 upwind, 4 vertical, and 5 downwind).

If the rising cell mass gives off a gas, and the wind washes it to leeward then there will be a greater concentration of the gas downwind than upwind. If the gas tends to repel the fruiting body it will orient

upwind. There is, of course, the possibility that the fruiting bodies orient by some sort of rheotaxis, but then this would be a totally new orienting mechanism which would have no relation to the normal orientation in still air.

To test between these two hypotheses (rheotaxis and gas orientation), a series of tests were performed in which two fruiting bodies were placed less than 0.6 mm apart and the air current (from 30 to 100 cm/sec) was passed between them. Out of 14 cases, 3 showed no repulsion, 4 showed slight or doubtful repulsion, and 7 showed the normal repulsion expected between two fruiting bodies standing close together. This may be compared with the same experiment in still air where all 18 cases showed definite repulsion. Since in some cases in the wind there was no repulsion between two closely positioned fruiting bodies, we may conclude that the gas was removed sufficiently between them so that they were unaware of one another's presence. This would argue against the rheotaxis hypothesis and support gas orientation, but then there are perhaps too few successful cases to warrant drawing any firm conclusion.

*Culmination under oil.* If the orientation is controlled by a diffusion gradient of a gas, then it would be expected that culmination under oil would inhibit the orienting mechanism. Since the work of Potts (1902), it has been known that culmination occurs under mineral oil; therefore, a layer of Parke Davis Heavy Mineral Oil was poured over plates of various cellular slime molds (*Dictyostelium discoideum*, *D. mucoroides*, and *P. pallidum*) that were about to culminate. In every case the fruiting bodies were completely twisted and disoriented (Fig. 8). This was especially evident in the larger sorocarps. In the smaller ones, and in the basal portions of the larger ones, there was usually a moderately straight initial section (i.e., at right angles to the substratum). It might be assumed that the initial rise from the agar was properly oriented (and therefore not by gas orientation), but as soon as the cell mass was up into the oil it had no further means of direction control.

It was also possible to see in these oil-covered dishes that when two fruiting bodies were close to one another they did not repel one another, and furthermore, obstacles, such as agar blocks, had no influence on their orientation.

Dr. David Francis of the University of Wisconsin suggested we try another liquid that also permits fruiting: FC-43, an "inert fluoro-



chemical liquid" kindly supplied by the Minnesota Mining and Manufacturing Company. Again the fruiting bodies were completely disoriented as they culminated into this liquid. It should be added parenthetically that both these nonconducting liquids would presumably permit normal orientation if surface electrical charges were the cause of orientation.

*Confined space.* According to the gas orientation hypothesis, it is necessary to maintain a gradient of the gas, for otherwise there is no directional information available for the sorocarp. It was thought that possibly a sufficiently small chamber could be devised so that the total concentration of the gas would build up to such a level that gradient discrimination would become ineffective. On the other hand, the chamber could not be too small for otherwise culmination would be inhibited by a lack of oxygen.

The method employed involved taking a series of glass tubes of different diameters and lengths, sealing off one end with a flame, and pushing this small cap down on agar possessing one or more centers about to culminate. The ideal-sized tubing had an inside diameter of 3 mm, and then the agar inside it produced a moist chamber of approximately 21 mm<sup>3</sup>.

Under these conditions the sorocarps culminated but were quite disoriented (Fig. 5). Two sorocarps near one another did not repel each other, and sometimes, if they culminated, they leaned directly into the glass wall. If a larger chamber was used with an internal space of approximately 1 cm<sup>3</sup>, the orientation was normal and precise.

*Experiments with charcoal.* If a rising fruiting body gives off a gas then it should be possible to absorb the gas unilaterally with activated charcoal. Aggregation centers of *P. pallidum* about to culminate were removed on blocks of agar and placed at right angles on a large block of agar. Activated charcoal was then placed on the surface of the base block so that it came within approximately 0.1 to 0.4 mm of the aggregation center. This is basically the same experiment as having culmination occur in a right-angle corner except for the fact that one side is charcoal rather than both being agar. In 20 experiments the fruiting bodies were in no case repelled by the charcoal surface as they had been from glass and agar surfaces in previous experiments. In fact, the rising sorocarp would often bump into a hillock of charcoal and rise again from the charcoal at various angles (Fig. 6). By simply placing a hill of charcoal near rising sorocarps it was evident that they were

actually attracted by the charcoal (Figs. 9 and 10). This is consistent with the idea that the gas can be absorbed, and therefore removed, by the charcoal creating an orientation gradient toward the charcoal.

#### ATTEMPTS TO IDENTIFY THE GAS

Assuming the gas hypothesis is correct, attempts were made to discover the nature of the gas for this is obviously the most direct method of positively confirming the hypothesis. As will be shown, this goal has not been achieved although during the course of these studies some new and useful facts have emerged.

*Species specificity.* The first question was whether or not there was a species specificity, i.e., whether one species could repel only its own kind. A series of tests were made in which two aggregation centers were placed close to one another. One was always *P. pallidum* and the other was either *D. discoideum*, *D. mucoroides*, or *D. purpureum*. The mean distance between the two centers was 0.38 mm, the range extending from 0.15 to 0.46 mm. The centers were placed in the rectangular observation chambers and incubated at  $23 \pm 1^\circ \text{C}$  in the dark.

In all cases and for all species there was a definite repulsion. In 18 cases the *P. pallidum* was repelled at a mean angle of 17.3 degrees with a standard deviation of  $\pm 14.0$  degrees. This is comparable with the 18.1 degrees  $\pm 13.2$  degrees when *P. pallidum* repels itself at a mean distance of 0.33 mm.

It is interesting that the three species of *Dictyostelium* were repelled at an even greater angle by *P. pallidum* in these experiments. *D. discoideum* had a mean angle away from *P. pallidum* of 27 degrees (4 cases); *D. mucoroides*, an angle of 27 degrees (5 cases); and *D. purpureum*, an angle of 30 degrees (10 cases). It is impossible, at the moment, to interpret this difference.

However, for our purposes, the important point is that *P. pallidum* responded to these three species as it does to itself. If the *Dictyostelium* species had no effect on *P. pallidum*, then one would expect its angle of repulsion to be similar to that produced by a glass rod of similar diameter. Therefore, one may conclude that at least among the species tested there is no species specificity.

*Water vapor.* One of the most obvious gases to test is water vapor. It is well known, since the pioneer work of Errera (1893, 1906), that hydrotropism plays an important role in the orientation of filamentous fungi. Potts (1902) noted orientation of culminating sorocarps of

*D. mucoroides* toward the opening of the gap created by placing a microscope slide above some developing fruiting bodies and interpreted this as evidence for hydrotropism. Using a variety of different humidity conditions Bonner and Shaw (1957) were unable to demonstrate hydrotropism.

The problem was approached in a number of different ways. First of all, some of the methods of Errera were tried. He found that agate and soft iron would attract the filaments of *Phycomyces*, and he made a convincing case for the idea that these substances will slowly take up water over a long period of time and therefore serve as low-activity desiccators. With the slime molds, soft iron, agate, porous clay, glass, or quartz had no effect at all on the sorocarp orientation except the usual repulsion at close distances (as with wet agar).

Next, small glass wells were filled with  $\text{CaCl}_2$  solution (5 or 10% solution giving approximate relative humidity values of 98% and 92%, respectively). A plastic (Lucite) cover with a hole about 1 mm in diameter was attached over the well and an agar block with a single aggregation center was placed on it so that the sorocarp would fruit horizontally over the hole above the well. This was then sealed off in a plastic observation chamber and placed in the dark  $23 \pm 1^\circ \text{C}$ . The distance between the plastic cover and the sorocarp base was in all cases less than 0.8 mm, but it must be remembered that the surface of the  $\text{CaCl}_2$  solution through the hole was 1 mm or more farther away.

In 18 cases the mean deviation from the right-angle orientation was 1.6 degrees and the standard deviation was  $\pm 16.9$  degrees. In other words, the unilateral placement of a  $\text{CaCl}_2$  solution does not produce any significant orientation, although the amount of variability in orientation has increased under these mildly desiccating circumstances. (In comparing these with the 55 controls,  $t = 0.051$ , which means that they are clearly not significantly different.)

The final and most effective argument against a humidity gradient being operative in orientation comes from an exceedingly simple experiment. If fruiting occurs at a uniform, constant temperature, in a completely sealed off chamber, then, according to the kinetic theory of gases, the water vapor will soon reach an equilibrium of complete and uniform saturation. Glass rings were sealed onto slides with beeswax and petroleum jelly, and in the chambers were placed agar blocks containing two aggregating centers in close proximity. Each chamber was then carefully sealed with a glass coverslip with more beeswax



and paraffin. As a further precaution, this was placed in a larger moist chamber, which was sealed with petroleum jelly. The chamber was in turn placed in a black box in a temperature-controlled room. Theoretically, there could be no water vapor gradient inside such cells, yet in each case the normal repulsion was evident. Therefore, the gas involved must be something other than water vapor.

*CO<sub>2</sub>, NH<sub>3</sub>, and other volatile substances.* It should be said right at the outset that in no case was it possible to produce any orientation with any of these substances. However, negative results are always questionable in their meaning, a fact that is particularly true in this instance. Here we have not only to discover the correct substance, but then to introduce it in such a way that both the slope of the gradient and the absolute concentration of the substance is in the sensitive range for the organism. Remember also that we are concerned with a gaseous gradient occupying a space somewhat smaller than a cubic millimeter. The best procedure will be to describe briefly what has been done, which might provide some useful guides for other workers as to what to try next.

Various methods were used, the simplest of which consisted of placing a gas-emitting test object at distances either greater or less than 1 mm from an aggregation center of *P. pallidum*. A heap of *E. coli* and bits of leaves of *Begonia* and *Coleus* were tested in this way with no effect. Besides CO<sub>2</sub>, it may be assumed that these metabolizing test objects were giving off a number of other gases, including ethylene in the case of the leaves.

In another set of experiments an aggregation center was set on the edge of a hole 1 mm in diameter in a plastic shelf which covered a small well containing KOH. The assumption was that because the KOH would absorb the CO<sub>2</sub> a gradient of CO<sub>2</sub> might be produced, but there was no effect on the orientation of the fruiting body.

The largest series of experiments were done in special vessels: rectangular plastic tubes 3.5 × 4 mm in diameter and 10 cm long. The aggregation center on an agar block was pushed through a round hole in the side of the vessel so that the top of the agar was flush with the inside of the vessel. On each side of the aggregation center the lumen of the rectangular tube was occluded with cellulose sponge about 10 mm long to reduce convection while permitting diffusion. Wet filter paper was also placed along the wall beyond the sponges on each side and the ends of the tubes were stuffed loosely with cotton. Twelve

of these tubes were fit snugly into holes in two cardboard boxes  $3 \times 9 \times 12$  inches; that is, the 12 tubes bridged the two boxes. A trough of water was placed in the bottom of one box and a test solution was placed in the other. The whole apparatus was then placed at  $23 \pm 1^\circ\text{C}$  in the dark.

The following solutions were tested:  $\text{NH}_4\text{OH}$  in a weak concentration that produced a faint ammonia odor, and a stronger concentration that produced abnormalities in some of the fruiting bodies, as described by Cohen (1953); bakers' yeast in a 10% dextrose solution (actively bubbling  $\text{CO}_2$ ; a series of microorganisms kindly supplied by Professor F. H. Johnson of this department which produced various gaseous substances when fermented in a peptone-dextrose broth (*Bacillus* sp., which gives off aromatic vapors; *Proteus vulgaris*, which produces  $\text{H}_2\text{S}$ ; *Oidium lactis*, which produces  $\text{NH}_3$ ; and *Serratia marcescens*, which has a putrefactive odor). In another experiment some intact *Coleus* leaves were put into one of the boxes. In none of these cases was there any significant orientation. It should be added that both in the controls and the experimentals in this series the amount of variation from the vertical was very small. (For 29 controls the mean deviation from the vertical is 0.6 degrees and the standard deviation  $\pm 7.5$  degrees.)

It should again be emphasized that in each of these cases we are probably not producing the correct concentration or gradient to achieve a proper test. To emphasize this point, let us cite some experiments in which 20 or more fruiting bodies were developing within a well and above the well a test aggregation center was placed so that it would grow out horizontally over a 1-mm diameter hole in a plastic cover over the well (as in the KOH experiment described in the preceding section). The test sorocarp was unaffected by the slime mold vapors rising from the hole; these vapors apparently operate only at distances less than 1 mm. It is, of course, not known whether this is solely because the gas becomes too dilute at greater distances or whether it also breaks down and becomes inactive. Another way action at a considerable distance was examined was to pass moist air over a large number of developing fruiting bodies just before (3 cm away) it arrived at the test sorocarp, but as before the single sorocarp still leaned into the wind. Again one must assume that the conditions did not achieve the proper concentration or gradient to produce any noticeable effect. The only possible exception might be the cases where wind

passed over many fruiting bodies, then all the ones on the upwind side of the group will lean into the wind, while if there are any remaining vertical or that leaned downwind they will be on the leeward side of the group. In this case the gas producers are rather close to those downwind of them and may have affected the orientation of the latter.

#### CONCLUSION AND SOME REMARKS ABOUT BRANCHING AND ADAPTATION

The evidence that culmination in the cellular slime molds is gas oriented is substantial, but until the gas involved can be identified and reintroduced *in vitro*, gas orientation can be considered only a hypothesis.

There is one further point which concerns the application of this hypothesis to normal development in the two species of *Polysphondylium* which have whorls or branches of stalked sori. If there are two branches at a whorl they jut off at about 180 degrees from one another; if there are three branches they jut off at about 120 degrees; if there are four, the angles are about 90 degrees. The only consistent exceptions to this rule were observed in the case of *P. pallidum* developing in the wind tunnel. Frequently the branches would tend to lean into the wind, as well as the main stalk, and in one case where there was a whorl of three branches, the two closest to the upwind side actually fused to produce a common sorus with two stalks.

This orientation in the branched species, as well as the general mechanism of orientation for single-stalked species, has considerable adaptive advantages with respect to natural selection. Not only does it permit the sorus to be held out free in an air space, but it also tends, in those cases where there are numerous sori, to spread them out evenly over the available space. Furthermore it has been shown that during aggregation spacing is also achieved by the formation of territories (Bonner and Dodd, 1962), and the combination of these two mechanisms should ensure an efficient spreading of the sori, producing optimum conditions for effective spore dispersal.

Finally, it should be added that, regarding both mechanism and adaptive advantage, there is considerable resemblance between the repulsion described here and the repulsion described for germinating hyphae of *Rhizopus* in the so-called "staling reaction" which Stadler (1952) has shown to be caused by a diffusing substance given off by



the growing hyphae. The important difference is that the staling substance involves diffusion in a liquid whereas the repulsions in the slime molds and in the aerial portions of fungus mycelia presumably involve a gas.

Futhermore Samuel (1961) has shown that the vegetative amoebae of cellular slime molds tend to repel one another. But there is no indication that the substances or the mechanism involved in the sorocarp repulsion are the same as those that act between vegetative amoebae. There is also the interesting question whether the gas orientation could be in any way coupled with the acrasin activity, for it is known that rising sorocarps are actively producing acrasin (Bonner, 1949).

#### SUMMARY

Evidence is presented to support the hypothesis that culminating slime molds orient with respect to their environment by producing a gas to which they are sensitive and they orient away from regions of high concentration (negative chemotaxis). A number of observations are described to show the capabilities and the sensitivity of the orienting mechanism, but as preliminary attempts to identify the gas were unsuccessful, its existence remains hypothetical.

#### ACKNOWLEDGMENT

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## Evidence for a Substance Responsible for the Spacing Pattern of Aggregation and Fruiting in the Cellular Slime Molds

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WITH TWO PLATES

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### INTRODUCTION

IN the cellular slime molds, after the individual amoebae have finished their growth and depleted their food supply, they aggregate into centers. Each center ultimately produces a small fruiting body (a spore mass supported by a slender stalk) that rises into the air. In a previous study (Bonner & Dodd, 1962*a*) it was shown that the size of the aggregation territories remained constant for any one species under a given set of environmental conditions even though the density of the amoebae in the culture dish might vary considerably. From this it was suggested that one of the possible hypotheses might be that a center-inhibiting substance is diffusing outward from the first formed centers and its effectiveness is independent of the number of cells within a territory. In another study (Bonner & Dodd, 1962*b*) evidence was brought forth to support the notion that as the fruiting body rises into the air it orients with respect to the environment by producing a gas to which it is sensitive and it orients away from regions of high concentration. In the discussion of this second paper we made the point that the adaptive significance of these two phenomena are one and the same; they both tend to space the fruiting bodies, first by keeping the centers separate during aggregation, and then by keeping the sporocarps separate as they rise into the air. Together they produce optimum conditions for effective spore dispersal.

We would now like to present the evidence that spacing in aggregation and spacing in culmination are governed by a common factor which we have tentatively called the *spacing substance*. (This term is used in a general sense to include the possibility of one or more chemicals.) The two previous studies which at first appeared quite unrelated except for their adaptive significance now seem to be separate aspects of a single process.

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*The three major components of slime mold development*

There are at least three basic mechanisms which control the social existence, the development of the slime mold amoebae. In the first place there is the mechanism by which *acrasin* orients the amoebae by positive chemotaxis and is responsible for the aggregation pattern. Many details of this process are obscure, but the broad outlines are well established (for recent reviews of this subject, see Shaffer, 1962; Bonner, 1963). It is also known that *acrasin* is produced in later stages of development within the cell mass (Bonner, 1949) but we remain ignorant of its function during the migration and culmination stages.

The second component of development is *center formation*. The appearance of centers precedes the *acrasin* mechanism and, as Shaffer (1961*a*) has shown, the first sign of center formation is the appearance of a cloud: a dense mass of amoebae which Samuel (1961) found are slow moving compared to the ones outside of the cloud. Certain cells within the cloud become the actual focal point of the center. In *Polysphondylium violaceum* Shaffer (1961*b*) gave irrefutable evidence that it is a single cell which he termed the 'founder cell'. This has also recently been confirmed for *P. pallidum* by Francis (personal communication).

The third component is the spacing substance. Arndt (1937), as well as numerous workers since, showed that early centers may reverse their progress and disintegrate, to use Shaffer's term. Recently Shaffer (1961*a, b*) demonstrated that founders may be inhibited and revert to ordinary cells. Furthermore he has made the interesting observation (Shaffer, 1962, 1963), which fits in perfectly with the argument to be presented here, that if a sandwich is made of pre-aggregating cells of *P. violaceum* over some aggregating ones, the new centers form in the regions of low cell density. From this he argues that there must be a diffusible center inhibitor which exists in a gradient highest near the old centers. In the present study we should like to show that a diffusible inhibitor exists for all the species tested, and that it is a major control mechanism in the spatial distribution and density of the centers which ultimately develop into fruiting bodies.

With these three mechanisms one has a method of producing centers of attraction, a method of seeing that the number and distribution of these is controlled, a mechanism of gathering the amoebae to the established centers, and finally a mechanism for preserving this spatial arrangement during the remaining period of development, right to the end of culmination.

## METHODS

In a few of the experiments the slime molds were grown in Petri dishes on non-nutrient agar upon which a generous loopful of *Escherichia coli* was evenly spread over the surface. The spores were inoculated at one point in the

center and allowed to grow and develop radially (Shaffer's modification of Singh's (1946) technique).

In the majority of the experiments the amoebae were grown first and then washed free of the remaining bacteria by centrifugation in standard salt solution (Bonner, 1947). Two methods were used in growing the amoebae; in some cases they were grown in Petri dishes containing nutrient agar (Peptone, 10 g., dextrose, 10 g.,  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$ , 0.96 g.,  $\text{KH}_2\text{PO}_4$ , 1.45 g., agar 20 g., distilled  $\text{H}_2\text{O}$ , 1000 ml.). At the time of the plates were inoculated with the spores and the *E. coli*, a few ml. of sterile distilled water was added. These were ready for harvesting after 2 days at 23°C. The other method used was that of Gerisch (1959, 1960, slightly modified by Hohl & Raper, 1962). Basically it consists of growing the bacteria first in liquid culture, washing them free of nutrient, and re-suspending the bacteria in Sorensen's buffer. The spores of the slime mold were added to this liquid culture and put on a shaking machine. One advantage of this method, as Gerisch (1962) has shown, is that it is possible to age amoebae in this way for all aggregation will be inhibited during the shaking process.

All experiments were run at  $23^\circ \pm 1^\circ\text{C}$ . in the light (constant ceiling fluorescent lights).

#### THE ORGANISMS AND THEIR AGGREGATION CHARACTERISTICS

One of the most interesting and often the most disheartening, aspects of development of the cellular slime molds is their extraordinary variability, not only among species, but within any one clone. Even under ideal controlled conditions aggregation patterns may take on variable characteristics. In the following brief description of the five species used, particular phenomena will be emphasized, but probably all of these species can show all the phenomena to varying degrees, and it is merely a matter of emphasis. Except for one phenomenon, none of these observations are original but have been noted by one or more previous workers.

*Dictyostelium discoideum* (Strain No. 1) (Plate 1). Usually this species has very long streams on which subsequently there may appear secondary centers. It is as though all the centers do not initiate at the same instant, but slowly over a period of time. It is even possible to see in *a* that some centers have already formed before general aggregation has occurred. Also note that some of the secondary centers which form on streams are subsequently attracted inward to the main center.

*Dictyostelium purpureum* (Strain No. 2) (Plate 1). Besides providing a good demonstration of clouds, this species shows a new and interesting phenomenon. If two centers arise close together, and they both retain their power as centers, then they move away from one another to a small but definite extent. This occurs even before any signs of migration or stalk formation.



*Dictyostelium mucoroides* (Strain No. 11) (Plate 2). In this series one may see the well known phenomenon of center suppression or dominance. A number of small centers appear which ultimately disband and join the larger centres.

*Polysphondylium violaceum* (Strain No. 6, Shaffer's original 'founder' strain) (Plate 2). This species consistently shows the most perfect and ideal spacing pattern. Note that one of the centers divides into two. For this species and others, this phenomenon becomes more common as the amoeba density is increased.

*Polysphondylium pallidum* (Strain No. 4). This species is not illustrated for it shows no additional characters that have not already been illustrated. Suppression of secondary centers is often very marked, and the streams of the aggregating amoebae tend to be symmetrical, like the spokes of a wheel.

#### *Evidence for a substance that inhibits aggregation*

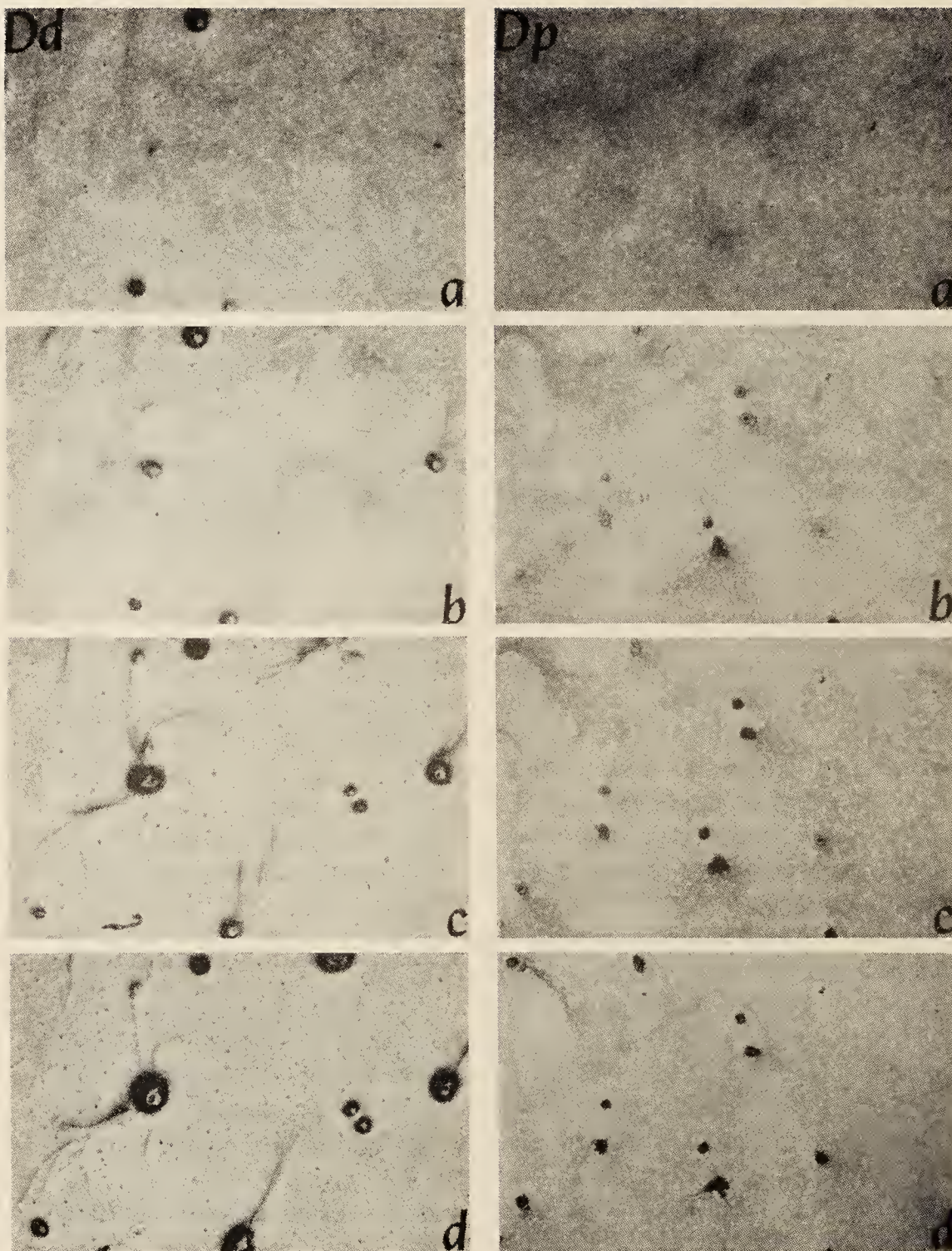
It has been noted on previous occasions that *D. mucoroides* frequently did not develop at all in confined conditions. If, for instance the amoebae were dispensed (after centrifugation) on non-nutrient agar in small plastic culture tubes stoppered tight with a cork (Falcon No. F25, 25 ml.) they frequently did not show any signs of aggregation, or if they did the aggregates were few and unhealthy in appearance. We have always assumed that this was caused by a lack of oxygen, for we know from the work of Gregg (1950) that aggregation is an aerobic process.

To test the oxygen deficiency hypothesis a set of stoppered culture dishes in which total inhibition of aggregation occurred (for about 12 hr. after the control with cotton plugs) were unstoppered and in some a few grams of activated charcoal were added, while nothing was added to the others. Each was again stoppered and placed at 23°C. The agar was on the upper surface of the tissue culture dishes so that the charcoal at the bottom of the dish lay about a centimeter away. In the controls without charcoal no aggregation occurred at all (they were observed for about 48 hr.) while in the dishes with the charcoal, after an hour, beautiful, normal aggregation occurred and vigorous, healthy fruiting was soon completed. Presumably, then, the inhibition is not caused by the deficiency of a gas (oxygen) but by the accumulation of an inhibitory gas which is effectively removed by the charcoal.

It was also found that aggregation and culmination would proceed in stoppered vessels if mineral oil (Parke-Davis, Heavy) was added. This could either be added, as with the charcoal, in the bottom of the dish, not touching the agar, or by filling the entire air space with oil. Therefore mineral oil is also capable of removing the inhibitory gas.

It is assumed that the amount of the gas produced is proportionate to the number of amoebae present in a tissue culture dish. To test this hypothesis stoppered dishes without charcoal were supplied with different numbers of





## EXPLANATION OF PLATES

Each of the photographs on the two plates represent an actual area on the Petri dish of  $6.3 \times 4.2$  mm.

## PLATE 1

*Dd, a-d.* A series of photographs of aggregation of centrifuged *D. discoideum* amoebae. The original amoeba density was 1820 amoebae/mm.<sup>2</sup>. The final density of the centers was 36 centers/cm.<sup>2</sup>. Each photograph is taken exactly 1 hr. apart. Note the attraction of centers that form in some streams to the large center (left of the middle) and the appearance of centers on streams (e.g. right of the middle). Also note that the centers do not arise at the same time.

*Dp, a-d.* A series of photographs of aggregation of centrifuged *D. purpureum* amoebae. The original amoeba density was 2020 amoebae/mm.<sup>2</sup>. The final density of the centers was 37 centers/cm.<sup>2</sup>. The time between *a* and *b* is 1 hr., while 30 min. have elapsed between *b-c* and *c-d*. Note the appearance of clouds and the fact that the centers which lie close together move apart slightly.

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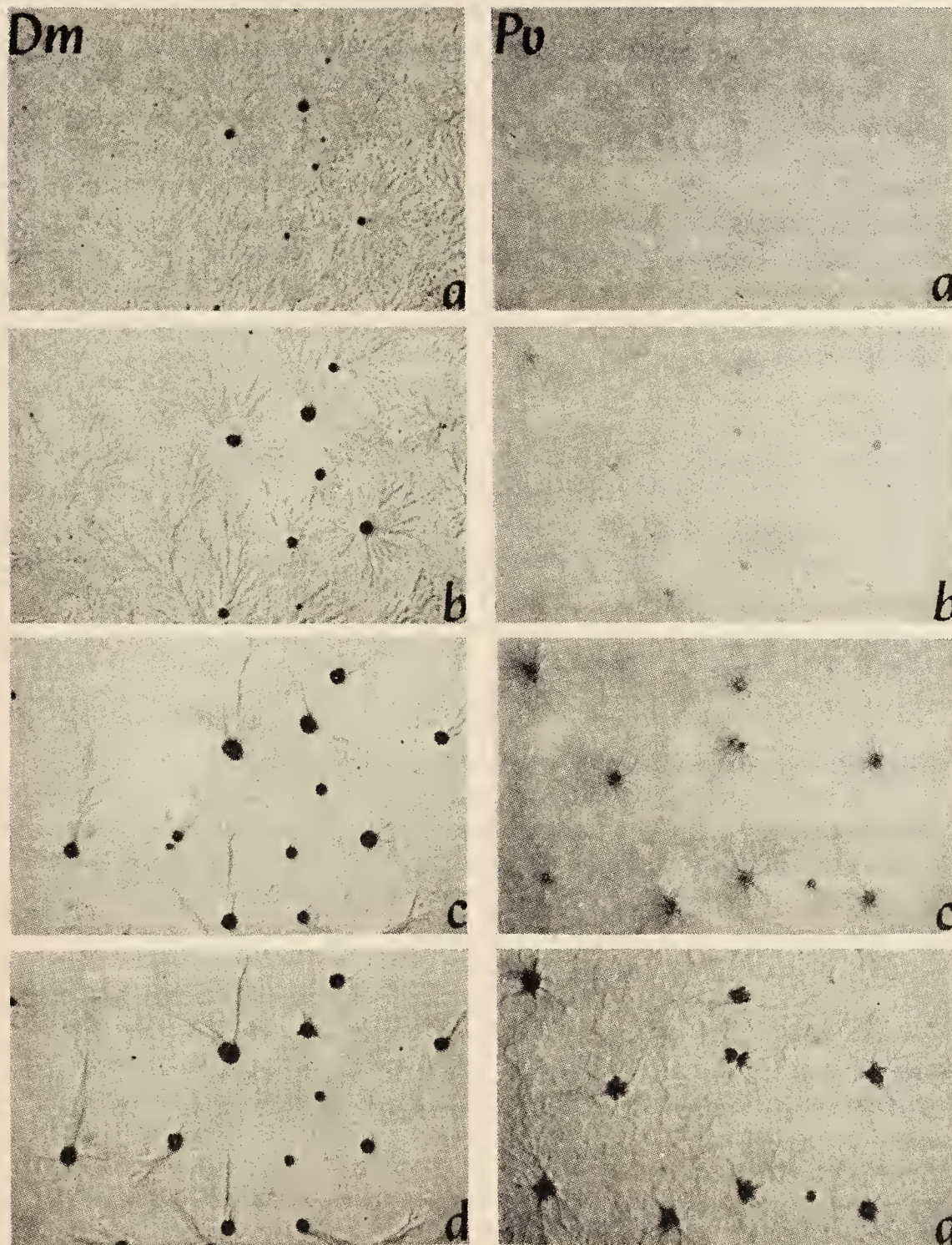


PLATE 2

*Dm*, a-d. A series of photographs of aggregation of centrifuged *D. mucoroides* amoebae. The original amoeba density was 1630 amoebae/mm.<sup>2</sup>. The final density of the centers was 41 centers/cm.<sup>2</sup>. The photographs are taken 30 min. apart. Note the disintegration of a number of the small centers which appear in *a*, and also note in comparing *c* and *d* (left of the middle) that a small center has joined a larger one.

*Pv*, a-d. A series of photographs of aggregation of centrifuged *P. violaceum* amoebae. The original amoeba density was 1120 amoebae/mm.<sup>2</sup>. The final density of the centers was 36 centers/cm.<sup>2</sup>. *a-b* is a 30-min. interval, while *b-c*, *c-d* are 1 hr. each. Note that the appearance of the centers is particularly well synchronized and that in one case (*d*, middle) a center divides into two.

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amoebae. In a series of experiments the range of cell densities was from 21,500 to 1200 amoebae/mm.<sup>2</sup>. Since the agar surface area is known (1653 mm.<sup>2</sup>) as well as the total air space within the dish (24 cm.<sup>3</sup>) it is possible to calculate the number of amoebae per mm.<sup>3</sup> of air space. Aggregation and fruiting occurred at the following cell densities 86, 126, 146, 250 amoebae/mm.<sup>3</sup> and total inhibition occurred at 236, 415, 546, 638 and 1479 amoebae/mm.<sup>3</sup>. Even though these ranges overlap they show that if the number of amoebae is greater than roughly 250 amoebae per cubic mm. of air space, the inhibitor they produce rises to a concentration where it is totally effective. This is the threshold of self inhibition.

It should be added parenthetically that under threshold conditions or conditions which in general favor the accumulation of the inhibiting substance, many familiar abnormalities appear, such as large aggregation swirls, that often have no centers but whirlpool-like central areas (Arndt, 1937; Raper, 1941); rounded humps after aggregation that stop completely in their development; all sorts of odd forms during migration and culmination including total disintegration (Shaffer, 1957). All of these well known deformities can be dramatically and quickly cured by a spatula full of fresh activated charcoal.

*Evidence that the gas inhibiting aggregation is a spacing substance*

Continuing with experiments on *D. mucoroides* it is possible to choose conditions in which the air space and the ventilation is sufficient so that development will occur without the help of activated charcoal and then compare these to similar preparations in which charcoal has been added. Both Petri dishes and plastic tissue culture dishes with cotton or plastic plugs (which allow some air passage) were used and the results have been summed, for there is apparently no significant difference between the two. In the case of the dishes with charcoal it was again placed in the bottom of the dish, some distance away from the agar on the upper surface. In nineteen controls the mean number of centers per cm.<sup>2</sup> was  $58 \pm$  a standard deviation of 77. In ten dishes with charcoal the mean was 246 centers/cm.<sup>2</sup>  $\pm$  234. Clearly if the gas is removed by the charcoal the territories are smaller and therefore we may assume that the greater the amount of inhibitor, the larger the territory.

If the agar is submerged in mineral oil then the mean fruiting body density is 1352 centers/cm.<sup>2</sup>  $\pm$  1690. Again one might assume that the oil, which is in contact with the amoebae, is even more effective in removing the inhibitor. But then the oil may have other effects as well since the gas phase has been totally removed. It should be added parenthetically that one of these effects might be dessication, for, as Raper (1940) showed, a lowering of the humidity produces smaller and more numerous fruiting bodies. In all experiments reported here great care was taken to avoid drying conditions.

Another brief but revealing experiment further confirmed that the inhibitory



gas is a spacing substance. Open Petri dishes containing the amoebae of *D. mucoroides* were placed in a desiccator jar lined with wet toweling. Moist air (passed through a sintered glass bubbler in a water trap) was circulated through the jar by means of a small aquarium pump. The control Petri dishes were in a similar jar in still moist air with their covers off. In two experiments the controls were 63 and 63 centers/cm.<sup>2</sup> while in the circulated air the values were 210 and 186 centers/cm.<sup>2</sup>. Presumably the circulating fresh air has blown away enough of the spacing substance to significantly reduce the territory size. To test the possibility that this might be due to desiccation, a gas insensitive species (to be explained presently) was used. *D. purpureum* gave the following values: 41 and 25 centers/cm.<sup>2</sup> in control conditions and 33 and 43 centers/cm.<sup>2</sup> in circulating air. Since drying affects territory size for this species (as well as all others), and since the controls and the experimentals are clearly comparable, the air circulation does not produce any drying effect on territory size. In one final experiment another gas sensitive species was also tested, and *P. pallidum* showed 14 centers/cm.<sup>2</sup> in the controls and 128 centers/cm.<sup>2</sup> in circulated air.

It should be added that in all the experiments where there is a reduction of the spacing substance, either by charcoal or air circulation, the aggregation starts somewhat sooner (usually  $\frac{1}{2}$  to 1 hr. sooner). Therefore the spacing substance would appear to delay aggregation, the extreme case being total inhibition in closed vessels.

At the suggestion and with the help of Dr David Francis tests were run to see if the distribution of centers in space was random or non-random, the latter being expected if the spacing substance is actively suppressing center formation.

Using the test of Clark & Evans (1954) it is possible to show that the distribution of centers is in all cases non-random. Also a series of dots were plotted on a graph by using random numbers obtained from the tables of Fisher & Yates (1943). If the distance between any dot and its nearest neighbor is plotted in a frequency distribution the resulting curve is approximately normal, while if the same is done for the distribution of aggregation centers, the curve is definitely skewed in such a way as to indicate that very short distances between centers are less frequent in the slime mold patterns than one would expect on a random basis. This is consistent with the notion that the centers are producing the spacing substance that inhibits new centers from forming in their immediate vicinity.

Also some experiments were performed in which two agar surfaces containing amoebae were separated from one another by a 5 mm. air space. It was found that the range of distances between centers and their nearest neighbor were the same for those cases in which the nearest neighbor was on the same agar surface as those where it was on the opposite agar surface (i.e. the diagonals across the air gap between centers). In other words for *D. mucoroides* the spacing substance can act as effectively across the gap as it can through or along the agar surface.

*The chemical nature of the spacing substance*

The first and the most crude experiment was one which indicated that there is no evidence for species specificity in the gaseous spacing substance. The experiment consisted merely of placing agar both on the bottom and on the top of a Petri dish. In the experiments two species confronted one another and in the control one species was confronted with cell-free agar. The result was that in seven experiments for *D. mucoroides* the controls showed a mean of 146 centers/cm.<sup>2</sup> while the summed experimentals (*D. mucoroides* confronted with *D. purpureum* in four cases and *D. discoideum* in three cases) showed an average of 99 centers/cm.<sup>2</sup>. Perhaps it would be more helpful to state that in four of the cases there was total inhibition with the partner and in the remaining three the number of centers/cm.<sup>2</sup> was reduced. Brief tests were also run with the two other gas sensitive species and *P. pallidum* (two cases) produced 211 centers/cm.<sup>2</sup> in the controls and 62 centers/cm.<sup>2</sup> when confronted with *D. purpureum*. Total inhibition was produced when it was confronted with itself (two cases). *P. violaceum* (two cases) produced 267 centers/cm.<sup>2</sup> in the controls and 130 centers/cm.<sup>2</sup> when confronted with *D. purpureum*. In the case of *D. discoideum* (three cases) and *D. purpureum* (eight cases) their territory size was clearly not increased by the addition of a partner producing additional gaseous spacing substance on the opposite side of a Petri dish. Thus as far as could be determined from these limited experiments all species produce the gaseous spacing substance, but only three of the five species are sensitive to it.

Partly inspired by the interesting observations of Loomis (1959) on the effect of CO<sub>2</sub> on sexual differentiation in hydra, we attempted some experiments in which specific CO<sub>2</sub> absorbants (KOH and diethanolamine) were placed in the bottoms of plastic tissue culture dishes (as in the charcoal experiments). To rule out the possibility of desiccation effects, in controls H<sub>2</sub>SO<sub>4</sub> was added at a concentration to give a relative humidity of approximately 95 per cent., which was slightly drier than that produced by 5 per cent. KOH (97 per cent. RH), but the number of centers/cm.<sup>2</sup> was comparable with the control. Also some of the tissue culture dishes (unstoppered or with cotton plugs) were placed in a desiccator jar containing moist air. Through a side opening alveolar air was blown in and then the jar sealed off (for the method of obtaining alveolar air see Best & Taylor, 1961, p. 486). This produced a CO<sub>2</sub> concentration inside the jar of over 5 per cent.

The results of these experiments are shown in Table 1 and also included are the previously mentioned experiments with charcoal and oil. It is obvious that conditions which allow a high concentration of CO<sub>2</sub> either increase territory size or result in total inhibition of aggregation, while the reverse is true when CO<sub>2</sub> is specifically adsorbed. The situation is not quite so clear cut in the case of some experiments on *P. pallidum*; although the added alveolar CO<sub>2</sub> did reduce fruiting body density from 96 centers/cm.<sup>2</sup> (corked control) to 26 centers/cm.<sup>2</sup>

(five cases), the  $\text{CO}_2$  absorbants gave results comparable in magnitude to the  $\text{H}_2\text{SO}_4$  controls, indicating that the desiccating effects are obscuring any possible  $\text{CO}_2$  deficiency effects. The territory sizes of *D. discoideum* and *D. purpureum* are unaffected by either the addition or the absorption of  $\text{CO}_2$ .

TABLE 1

*Number of centers/cm.<sup>2</sup> in D. mucoroides developing in different gaseous environments*

Condition	Centers/cm. <sup>2</sup>	No. of cases
Alveolar $\text{CO}_2$ added	0	4
Corked control	33	7
Open control	58	19
5 per cent KOH	134	6
60 per cent Diethanolamine	136	3
Activated Charcoal	246	10
Mineral oil	1352	10

A few runs were made on a Beckman gas chromatograph (Model CG-2) with the help of Mr R. Hyde. Gas samples from tissue culture dishes containing large concentrations of *D. mucoroides* amoebae were analyzed using a 6 ft. silicon column and these samples differed from air only in that the one large peak (which includes  $\text{CO}_2$ ) is slightly broader in the slime mold samples.

These preliminary experiments are consistent with the idea that  $\text{CO}_2$  could be the gaseous spacing substance. But it is also possible that besides  $\text{CO}_2$  there are other significant gaseous substances. Furthermore it is possible that  $\text{CO}_2$  has effects which are merely similar to the normal gaseous spacing substance. These possibilities have not been rigorously ruled out and must be before we can reach any final conclusion.

There is also the puzzling fact that some species are completely insensitive to the gas yet they clearly do have spacing and a non-random distribution of aggregation centers. In all the species it is possible to produce aggregation and demonstrate territory formation under a layer of agar, or in the bottom of a glass dish under a layer of standard salt solution (Bonner, 1947). The territory size under these conditions is roughly the same as on the surface of agar. Therefore in all species the spacing substance can diffuse through an aqueous medium and a gas phase is not required. In the gas sensitive species this could still be  $\text{CO}_2$ , but this would not account for territory formation in the gas insensitive species. It was thought that perhaps in these latter species they were sensitive to bicarbonate and therefore a series of tests were run both underwater and on agar surfaces at different pH's. Unfortunately the results are thus far confusing and contradictory and require an extensive and systematic study which we hope to undertake. All that can be said at the moment is that changes in pH do have a very marked if inconsistent effect on aggregation territory size. It is for all these reasons that it still seems advisable to use the term *spacing substance* in the general sense of being possibly one or more chemicals.



*Variations in the aggregation patterns*

As already emphasized, the variations in aggregation patterns are considerable and now our problem is to attempt to understand these variations. First a series of observations will be described with the hope that they will provide some basis for interpreting the underlying causes.

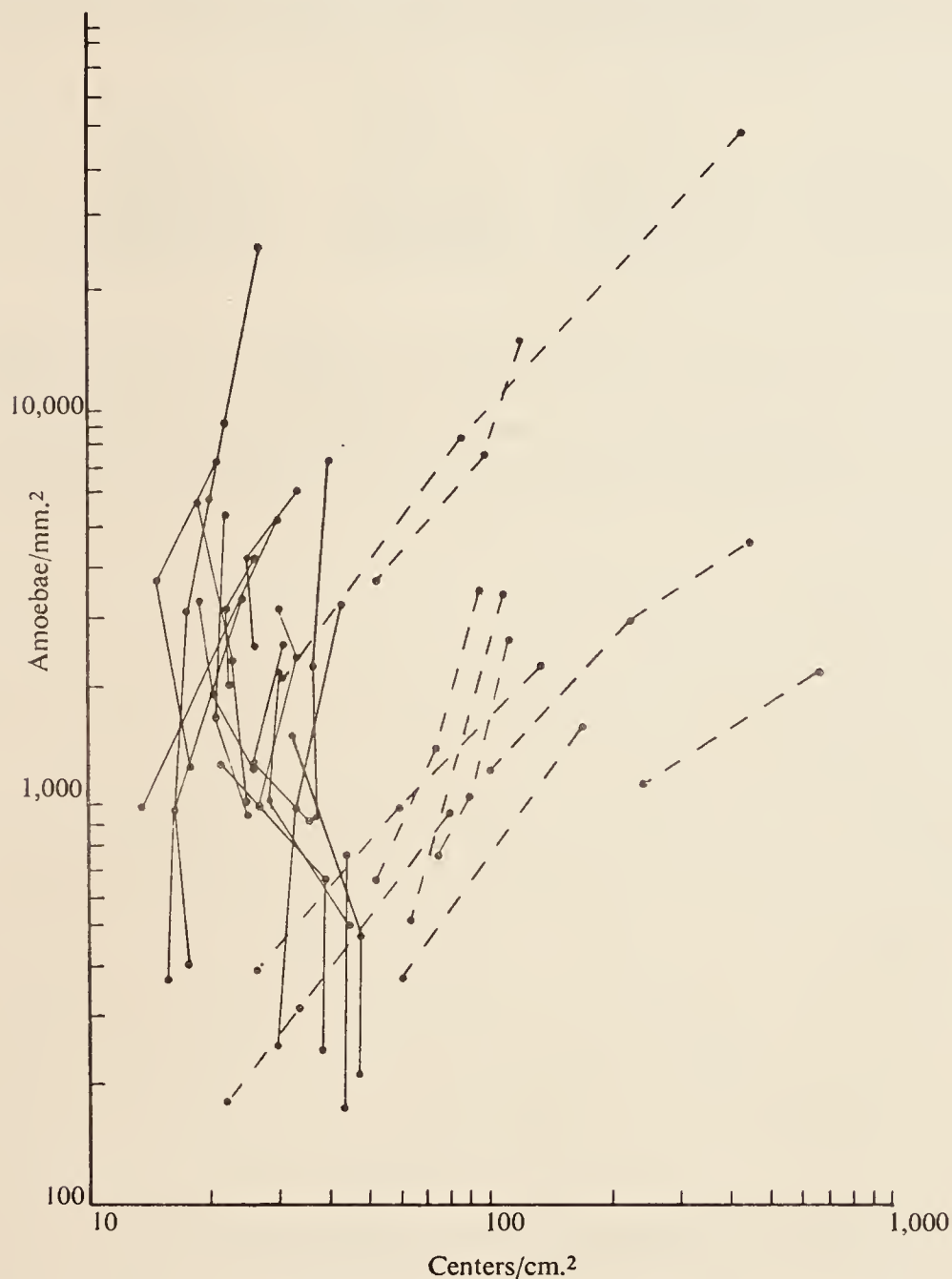
In a previous study on aggregation territories (Bonner & Dodd, 1962a) we were very fortunate in doing the majority of these studies with growth plates, for such preparations give remarkably constant territory size over a great range of amoeba densities. In the last year and a half we have run many hundreds of tests with all five species using centrifuged amoebae, and we now find that the only species that holds this amoeba density relation completely with centrifuged amoebae is *D. purpureum* (see the solid lines in Text-fig. 1). In *D. discoideum* there is a definite decrease in territory size as amoeba density increases. In *D. mucoroides*, and particularly *P. violaceum* and *P. pallidum*, there is so much variation that a definite relation requires too much imagination to perceive with any confidence. The question is how to explain these differences.

One way in which the species differ is in their susceptibility to the spacing substance, as has already been discussed. This is shown in summary in Table 2 and it can be seen that as one proceeds down the list the species are progressively more affected by the conditions which remove the spacing substance (oil and charcoal). In other words *D. purpureum* which is so especially rigid in producing fixed territories under all conditions is especially insensitive to the volatile spacing substance, and this correlation may be of significance.

The other important feature, which is obscured by the averaging in this table, is the fact that *P. violaceum* apparently can exist in two states. It alternates through periods of relative insensitivity to periods of great sensitivity. *D. purpureum* and *D. discoideum* are at all times relatively insensitive and *D. mucoroides* and *P. pallidum* are at all times sensitive to the spacing substance. *P. violaceum* seems to possess both characters which switch on and off in succession. The causes of the switching are not understood.

Another feature which has been studied in some detail is the reversibility of center formation. Two extreme cases were chosen; *D. purpureum* and *P. pallidum*, and for each their amoebae were allowed to begin aggregation and then were flooded with standard salt solution, stirred, filtered through cotton, and re-suspended on fresh agar. In both cases (the dotted lines in Text-figs. 1 and 2) the number of centers formed became density dependent, as shown by the fact that the lines have a slope of 1 on a logarithmic scale. In other words, to examine *D. purpureum* first (Text-fig. 1), if the amoeba concentration is halved, the number of centers is halved, which is contrary to what happens when aggregation occurs for the first time and the territory size is fixed at all amoeba densities. Once a center of *D. purpureum* is formed and territories have been blocked out, the process is irreversible, at least under these circum-

stances. The centers can be fragmented and each one produces many new centers upon replating, but they do not lose their tendency to produce centers.



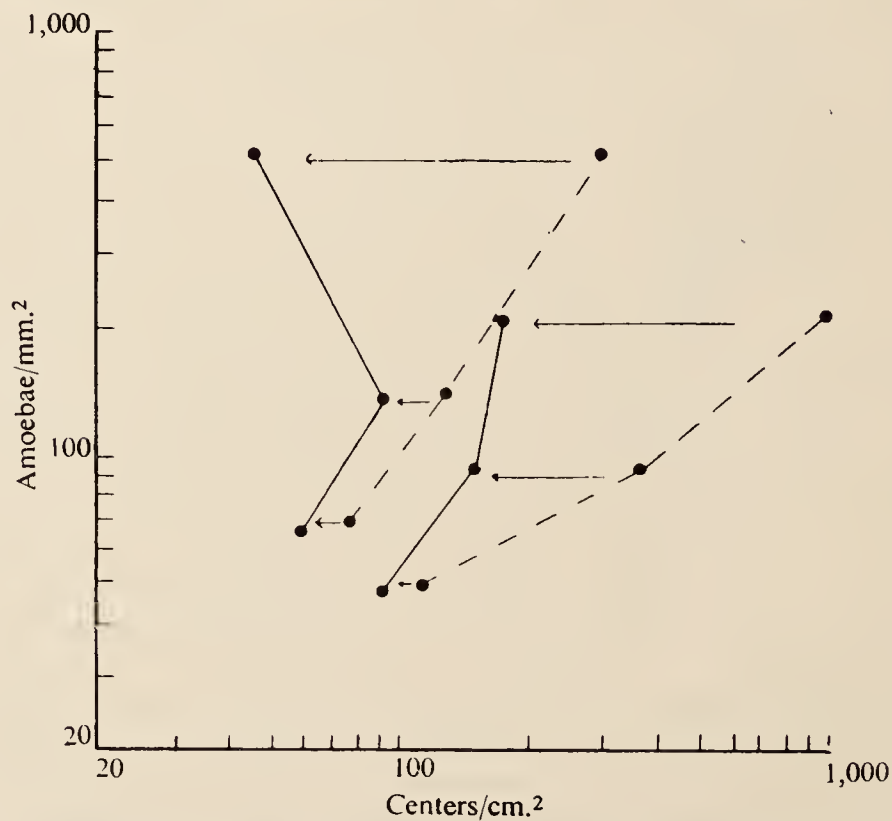
TEXT-FIG. 1. The log of the amoeba density (amoeba/mm.<sup>2</sup>) plotted against the log of the aggregate density (centers/cm.<sup>2</sup>) for *D. purpureum*. The solid lines are of amoebae taken directly from shaker flasks (modified Gerisch (1959), technique) while the dotted lines are of amoebae that have aggregated once and have been partially separated and replated on fresh agar. Each line represents one experiment with successive dilutions of the amoebae. If the line is vertical this indicates that center formation is independent of the amoeba density; if the line is at 45° then if the number of amoebae is halved, so is the number of centers (i.e. dependent on the amoeba density).

TABLE 2

Number of centers/cm.<sup>2</sup> under different conditions for five species  $\pm$  standard deviations. Numbers in brackets are the number of cases

	Air	Air with charcoal	Under oil
<i>D. purpureum</i>	35 $\pm$ 14 (22)	50 $\pm$ 14 (5)	58 $\pm$ 27 (19)
<i>D. discoideum</i>	75 $\pm$ 71 (13)	111 $\pm$ 122 (7)	90 $\pm$ 82 (9)
<i>P. violaceum</i>	130 $\pm$ 182 (32)	762 $\pm$ 695 (9)	1100 $\pm$ 2520 (25)
<i>D. mucoroides</i>	58 $\pm$ 77 (19)	246 $\pm$ 234 (10)	1352 $\pm$ 1690 (10)
<i>P. pallidum</i>	80 $\pm$ 66 (24)	345 $\pm$ 94 (3)	3043 $\pm$ 5840 (22)

The whole mechanism of territory formation and suppression can no longer operate. The only exception to this rule for *D. purpureum* is in underwater preparations where after a day or so the first centers (which cannot develop further under water) will disintegrate and form new ones with larger territories.



TEXT-FIG. 2. The log of the amoeba density (amoeba/mm.<sup>2</sup>) plotted against the log of the aggregate density (centers/cm.<sup>2</sup>) for *P. pallidum*. In these two cases cells in the process of aggregation were partially separated and resuspended on fresh agar. At first the centers appeared density dependent (dotted lines) but after about 8 hours many of the first formed centers were suppressed producing roughly the same number of centers over a wide range of amoeba densities (solid lines).



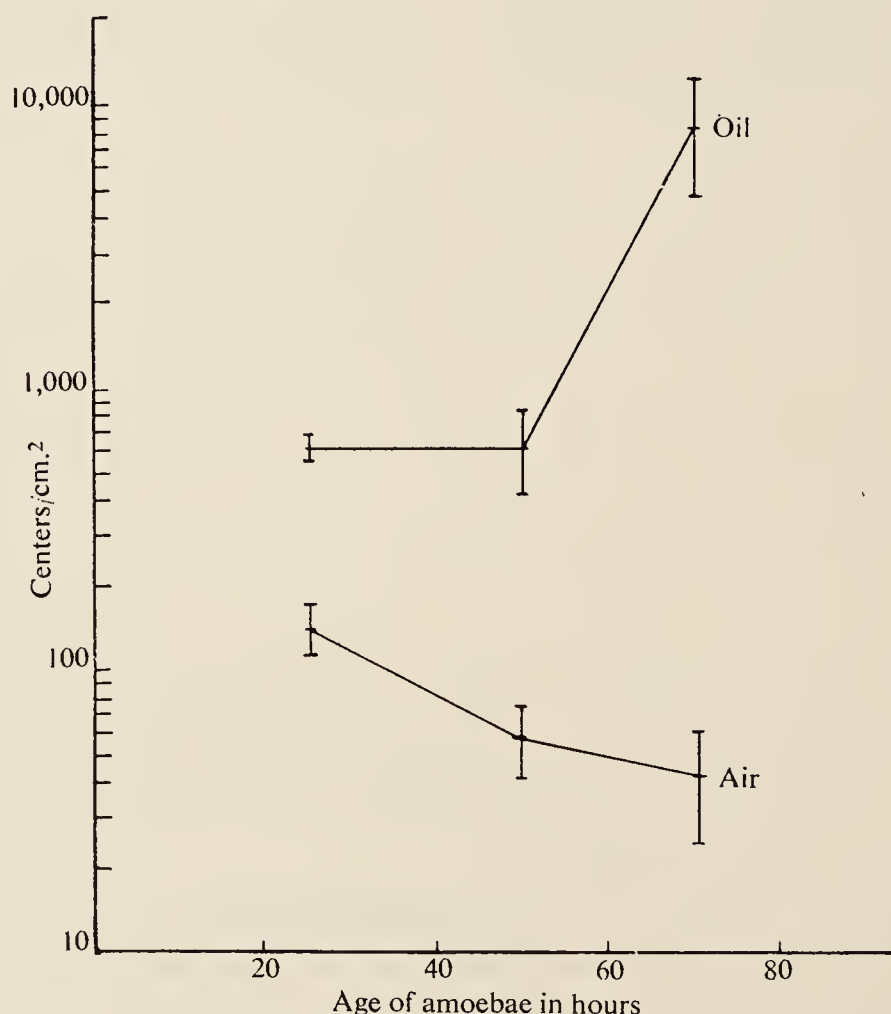
*P. pallidum* is far more fickle, for in a matter of approximately 8 hr. many of the new centers are suppressed so that the number of centers/cm.<sup>2</sup> reverts from a density dependent to a density independent condition (Text-fig. 2). Perhaps sensitivity to the spacing substance and center reversibility are correlated phenomena.

Center sensitivity might also account for another phenomenon observed previously (Bonner, 1960). It was found that if very small groups of cells were isolated on a growth plate by scraping away the cells immediately about them, then their success in completing development depended upon the number of cells in the group. In the case of *D. mucoroides* (Fig. 1-4, Bonner, 1960) if there were slightly over 100 cells in a group its chances of culminating rather than disintegrating were 50 per cent. In the case of *D. purpureum* (Figs. 1-5) dispersal was almost impossible to achieve unless the centers were very small (*ca.* 25 cells) but culmination was abnormal unless the number of cells in the center was fairly large. On the other hand Konijn & Raper (1961) showed that in isolated drops small centers never disintegrated nor formed abnormal fruiting bodies. We would now propose the hypothesis that in the growth plates (as compared to Konijn and Raper's preparations) there was an accumulation of the spacing substance from the surrounding amoebae and pseudoplasmodia and small groups of cells are particularly sensitive to it. In fact, the curves in Figs. 1-4 and 1-5 of the 1960 paper show the number of cells necessary to resist disintegration in the presence of a given but unknown concentration of spacing substance. This hypothesis is further supported by the fact that the types of abnormalities observed in *D. purpureum* culmination are now known to be cured by the addition of activated charcoal in the culture dish. It is also worthy of note that again *D. purpureum* shows greater resistance than *D. mucoroides* to the spacing substance.

There is another source of variations that can be demonstrated by using the Gerisch (1959, 1960) technique, as Gerisch (1962) himself has shown. It is possible to prevent the amoebae from aggregation by keeping them continuously on the shaker. In the case of *D. purpureum* it makes no difference when the amoebae are taken: they all give fixed territories, and as can be seen from Table 2 the territory size in air is roughly comparable to that under mineral oil. However, at the other extreme, 72-hr.-old amoebae of *P. pallidum* under mineral oil show an extraordinary increase in the number of centers/cm.<sup>2</sup> (Text-fig. 3). This is not true in air so we must assume that the spacing substance suppressed all these new centers, but that the mineral oil prevented the spacing substance from acting and therefore the new centers could express themselves without inhibition. There is no explanation for the increase in territory size with amoeba age in the air experiment, except possibly that with age the amount of spacing substance increases or the susceptibility to it increases.

The importance of timing is also shown in the normal development of *D. discoideum*. As can be seen from Plate 1, the centers in this species do not form

at one time (as they do, for instance, in *P. violaceum*, Plate 2); rather they appear slowly over a period of time. If the time at which a center is produced can vary as well as the timing of susceptibility which is illustrated (as already mentioned) in *P. violaceum*, then the timing alone would provide many possibilities in variation.



TEXT-FIG. 3. Graph showing the effect of age of the amoebae in the shaker (modified Gerisch (1959) technique) on the log of the number of centers/cm.<sup>2</sup> for *P. pallidum*. The upper line is under mineral oil, while the lower one is in air. The standard error is indicated for each point.

Another cause of variation can only be mentioned in passing for we have no specific experiments to report. This is the important effect of environmental changes. As already mentioned Raper (1940, 1962) showed that lowering the humidity decreased territory size. Potts (1902), Harper (1932) and Raper (1940) have also shown that an increase in light causes a reduction in territory size. More recently Shaffer (1958) has shown that a sudden onset of light will induce aggregation in some species, and specifically produce a spate of new founder

cells in *P. violaceum* (Shaffer, 1961*b*). In this study every attempt was made to reduce these variables.

In conclusion it may be stated that the variation in aggregation patterns have in general as their basis (1) variation in center formation, (2) variation in spacing substance production, and (3) variation in susceptibility to the spacing substance. On top of these one must add the activities of acrasin and how they follow through with aggregation. Each one of these aspects is in turn affected independently by the genetic constitution of the species or strain, the environment, and finally by the timing of the events. Considering all these possibilities it is perhaps hardly surprising that the possible permutations of pattern are considerable.

#### *The spacing substance and the later stages of development*

From the fact that the distribution of centers in all species is non-random we assume that all species produce and respond to a spacing substance and from the experiments in which one species confronted another there was evidence that during aggregation all species produced the gas form of the spacing substance, even those species that are insensitive to its effects during aggregation. Now arises the question of whether or not the substance continues to be produced by the migrating and culminating pseudoplasmodia.

The fact that it is continually produced during the later stages was demonstrated by a simple experiment. If a growth plate (i.e. non-nutrient agar covered with a thin layer of *E. coli*) is inoculated one at spot with a few spores of *D. mucoroides*, as the amoebae grow and eat their way outward radially there are continuously expanding concentric circles of successive stages of aggregation and fruiting. If, on the other hand, a huge mass of amoebae is placed at the inoculum spot of a comparable culture plate, then simultaneously the amoebae will spread and large fruiting bodies will arise from the inoculum spot over the vegetative and pre-aggregation amoebae. When this occurs, as long as the large aerial pseudoplasmodia are actively migrating or culminating, none of the amoebae on the surface of the Petri dish will begin aggregation even though their density and age be suited for aggregation. This suppression of aggregation will occur even when the rising pseudoplasmodia are not touching the agar surface (except for the bases of their stalks at the point of inoculation).

The adaptive function of such inhibition would appear to be the same as during aggregation, namely the prevention of cell masses from forming near others: the fact that the original group of cells is at a very much later stage of development does not alter the spacing action of the inhibitory substance. But inhibiting aggregation is not the only activity of the spacing substance produced during later stages of development.

Another is the curtailing of migration. This is especially obvious in *D. discoideum* where the migration stage is sharply defined. If two sets of stoppered plastic tissue culture dishes are inoculated with the same quantity of *D. discoideum*



amoebae, but in one group activated charcoal is added, then on the average migration lasts from 12 to 24 hr. longer in the presence of the charcoal than in the controls. In conditions which favor the accumulation of the spacing substance there is reduction of the duration of migration. (It should be noted parenthetically that during migration lowering the humidity produces the same effect as the spacing substance while they have opposite effects on aggregation, i.e. dryness causes aggregation to occur sooner (Raper, 1940). This is one more bit of evidence to support the fact that the spacing substance effects are quite distinct from the effects of desiccation.)

In a recent study Wescott (1960) was able to show that 5 and 10 per cent. concentrations of  $\text{CO}_2$  did not inhibit fruiting in *D. discoideum* but that 5 per cent.  $\text{CO}_2$  caused some reduction of migration, while 10 per cent.  $\text{CO}_2$  almost completely eliminated the migration stage. We have confirmed these results using alveolar air. This again is a case where  $\text{CO}_2$  has the same effect as the spacing substance.

The most important activity of the spacing substance during the last stages of development is orientation (Bonner & Dodd, 1962*b*). As the pseudoplasmodium rises into the air it apparently gives off a gas to which it is characteristically sensitive and it moves away from regions of high concentrations. This results in the repulsion of pseudoplasmodia rising less than 0.8 mm. from one another. Also solitary pseudoplasmodia orient with respect to the physical structures of their immediate surrounding space: normally the substratum is a flat surface and the pseudoplasmodium therefore rises perpendicularly, since this is the only position in which the gases will be of equal concentration on all sides.

The assumption that the gas responsible for the orientation of the cell mass is the same as that which inhibits aggregation is based upon the fact that neither are species specific and they are both adsorbed by charcoal and by mineral oil. The latter, in the case of orientation, was first observed by Shaffer (personal communication). If a small drop of oil is placed on the surface of agar both migrating and culminating pseudoplasmodia will curve right into it, as they do with the charcoal. It should be added that the repulsion shown between close aggregation centers (*D. purpureum* in Plate 1) might be another argument that the same spacing substance is present throughout development. If the spacing substance is  $\text{CO}_2$  this, of course, would be expected.

#### DISCUSSION

A question of prime importance is how the spacing substance acts. One hypothesis might be that it speeds the rate of movement of the amoebae. Center formation seems to be associated with the cessation of movement and therefore inhibition of center formation could be produced by stimulating the movement of the cells so that they break up from the groups. This would also explain the negative chemotaxis during culmination for by having faster move-

ment on the side of high concentration of the spacing substance, the cell mass would tend to curve away.

There are of course other possible explanations and furthermore it would not be surprising if the spacing substance did its center inhibiting by a different process than its orientating of the pseudoplasmodia. There is also the interesting question whether or not the mutual repulsion of the vegetative and pre-aggregating amoebae demonstrated by Samuel (1961) is also an action of the same spacing substance and what its mechanism of action might be in this case.

Finally it is also interesting to speculate as to the possible evolutionary origin of the cellular slime molds in the light of these observations on the spacing substance. We might assume that the spacing substance existed first, producing a mutual repulsion of the amoebae, a property which is apparently possessed by solitary soil amoebae. Center formation might have been the next evolutionary step; certain regions became ones of immobilization and there resulted a struggle between these centers and the spacing substance. The aggregation of cysts in the soil amoeba *Hartmanella* might be an example of this (Ray & Hays, 1954). If this congregation by immobilization has selective advantages, then if a mechanism were to arise which actively guided the amoebae inward, such as the acrasin mechanism, we would have the situation found in all the higher cellular slime molds. Acrasin has the further advantage of aligning the cells and from this polarized group of cells we find all sorts of new properties arising, especially oriented movement and differentiation.

#### CONCLUSIONS AND SUMMARY

The development of cellular slime molds is dependent upon three major factors; (1) the acrasin mechanism which gathers the cells together by positive chemotaxis, (2) the process of center formation which determines the locus of the gathering, and (3) the spacing substance that determines; (a) regions in which centers will be prevented from arising, and (b) inhibition of some centers that are already formed (i.e. the spacing of the aggregation centers): (c) the orientation or spacing of the fruiting cell masses.

Certain of the facts fit in with the hypothesis that the spacing substance is  $\text{CO}_2$ . However two species (*D. discoideum* and *D. purpureum*) always, and another (*P. violaceum*) sometimes, are insensitive during aggregation to both the naturally produced gaseous form of the spacing substance and added  $\text{CO}_2$ . Yet they do have non-random spacing during aggregation and therefore must have some form of substance diffusible in water.

There are great variations in the pattern of aggregation and these may be accounted for on the basis of variation in (1) center production, (2) spacing substance production, and (3) susceptibility to the spacing substances, and the changes with time of any of these three components for any one species under a particular set of environmental conditions.



During the later stages of development the pseudoplasmodium continues to produce the spacing substance. It has the effect of: (1) inhibiting a possible wave of secondary aggregation until the first wave of fruiting is completed; (2) inhibiting migration; (3) orienting the rising sorocarps of all species, for the pseudoplasmodia orient by negative chemotaxis in a gradient of the gaseous form of the spacing substance (Bonner & Dodd, 1962*b*).

## RÉSUMÉ

*Données relatives à la disposition spatiale des agrégats et des fructifications dans les myxomycètes en phase cellularisée*

Le développement des myxomycètes en phase cellularisée dépend de trois facteurs principaux; (1) le mécanisme dû à l'acrasine qui agglomère les cellules par chimiotactisme positif; (2) le processus formateur de centres qui détermine le lieu de l'agglomérat; (3) la substance espaçante qui détermine (a) les régions dans lesquelles des centres seront empêchés de se constituer; (b) une inhibition au niveau de certains centres déjà constitués (d'où un espacement des centres d'agrégation); (c) l'orientation et l'espacement des masses cellulaires en fructification.

Certaines des données s'accordent avec l'hypothèse que la substance espaçante est le CO<sub>2</sub>. Cependant deux espèces (*D. discoïdum* et *D. purpureum*) se montrent toujours indifférentes pendant leur agrégation à la fois à la forme de CO<sub>2</sub> qu'elles produisent naturellement et à celui que l'on ajoute, et il en est parfois ainsi également pour *P. violaceum*. Cependant, ces espèces ne présentent pas, pendant leur agrégation, un espacement qui soit l'effet du hasard et doivent donc disposer pour cela de quelque substance diffusible dans l'eau.

Il y a de grandes variations dans le dispositif d'agrégation et elles peuvent être interprétées en fonction de: (1) la production de centres; (2) la production de substance espaçante; et (3) la sensibilité à l'égard de ces substances et aux changements que, chez chaque espèce, la valeur de ces composantes subit au cours du temps dans un ensemble particulier des conditions de milieu.

Pendant les stades ultérieurs du développement, le pseudoplasmode continue à produire la substance espaçante. Elle a pour effets: (1) d'inhiber une vague possible d'agrégation secondaire jusqu'à ce que la première phase de fructification ait été menée à bonne fin; (2) d'inhiber la migration; (3) d'orienter le surgissement des sorocarpes de toutes les espèces car les pseudoplasmodes s'orientent grâce à un chimiotactisme négatif dans un gradient de la forme gazeuse de la substance espaçante (Bonner et Dodd, 1962*b*).

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# HOW SLIME MOLDS COMMUNICATE

Slime-mold amoebae are free-living microorganisms that periodically gather together to form macroscopic fruiting bodies. It now seems that secreted gases play several important roles in this development

by John Tyler Bonner

Living things generally grow "up" because the earth's gravity pulls them "down." A stand of pines thus points to the zenith from a hillside as well as from a flood plain. The tiny fruiting body of the cellular slime mold is also lofted upward into the air—by as much as two whole millimeters—above the surface from which its spindly supporting stalk happens to spring. Now, a great many questions remain to be answered about this peculiar organism. In the first place, it is formed by the intricate and transitory collaboration of a number of single-celled animals. The system of intercellular communications that brings these social amoebae together and causes them to differentiate in form and function and find their proper places in the rigorously ordered structure of their fruiting body holds clues to the developmental processes of multicelled plants and animals that arise in the usual way from a single cell. The fact is, however, that gravity does not supply any significant cue to the orientation of the growth of the microscopic slime mold. Presumably the weights involved here are too insignificant.

Not long ago in our laboratory at Princeton University we came on another principle of action at a distance that may explain the orientation of the upward reaching of the social amoebae. We were not at the time worrying about what the cells say to one another in the process of marshaling a unified multicellular organism. We had become interested in what might be termed the conversations between the cell masses and their neighbors. We had raised the level of discourse, in other words, from that of cells to that of organisms composed of numbers of cells. It now appears that the same principle of communication is engaged at both levels. Slime-mold amoe-

bae have turned out to be even more social than anyone had suspected.

Of the dozen or so known species of cellular slime mold, *Dictyostelium discoideum* has played the leading role in the laboratory ever since it was discovered and described by Kenneth B. Raper of the University of Wisconsin in 1935. In the free-living, single-celled state this amoeba has the size and appearance of one of our white blood cells. It feeds on bacteria in the moist humus where it dwells and, under favorable conditions, undergoes repeated division every three or four hours. By the time the local food supply is depleted there is a considerable accumulation of amoebae wandering about in different directions. Suddenly they shift their attention and begin streaming into central collection points. Much work has been done on this aggregation stage; it is known that an evanescent substance called acrasin is given off by the amoebae, and that the amoebae tend to move up the gradient of concentration of this substance, forming a clump of cells often visible to the naked eye [see "Differentiation in Social Amoebae," by John Tyler Bonner; *SCIENTIFIC AMERICAN*, December, 1959]. If the aggregation is sufficiently large, it will form a sausage-shaped slug that crawls about and orients toward sources of light and warmth with remarkable precision and sensitivity. Eventually the mass rights itself and the leading (now upper) third of the cells begin to differentiate into stalk cells. These cells form a central cylindrical stalk, stiffened by cellulose fibers that the cells secrete. The rest of the cells stream upward to the top of the stalk, where they form a little sphere, each cell encapsulated into a spore ready to start a new generation.

All sorts of intercellular communica-

tion play their parts in this curious life history. E. W. Samuel of Antioch College has shown that, before aggregation begins under the influence of acrasin, the amoebae tend to repel one another. Like sheep, they separate to graze; once the food is gone they come together. After aggregation the intercellular communication system regulates the differentiation and sorting out of the cells to form the stalk and fruiting body. Only a few features of this system have been demonstrated by experiment.

The first clear indication of communication between cell masses was uncovered by two college seniors working in our laboratory, J. Rorke and G. Rosenthal. They were investigating a phenomenon we first noticed in 1941. If a migrating slug is cut into three pieces, each piece will regroup and before long culminate, that is, rise into the air to form a small but otherwise normal sorocarp, or fruiting body. If the three pieces remain close to one another, they will tend to bend away from one another as they rise, instead of growing straight up. The readiest explanation appeared to be that the forward third of the slug grew faster and so pointed forward, whereas the slower growing hind third grew slower and pointed rearward. In one experiment, however, Rorke and Rosenthal noted that the forward fragment had wandered around and settled in position between the middle and hind fragments before the three fragments began to culminate. When they rose into the air, the two end cell masses still leaned away, whereas the new middle piece went straight up.

In other words, the orientation of the rising sorocarp has nothing to do with what part of the slug the cells come from. The only significant point



is their relation to one another as they rise. If they are close to one another, they will repel one another. We confirmed this deduction by trying all the different permutations of forward, middle and hind fragments, as in the shell game, and even got the same result when we pushed two complete and unrelated cell masses close to each other. In every case they leaned away from each other as they rose.

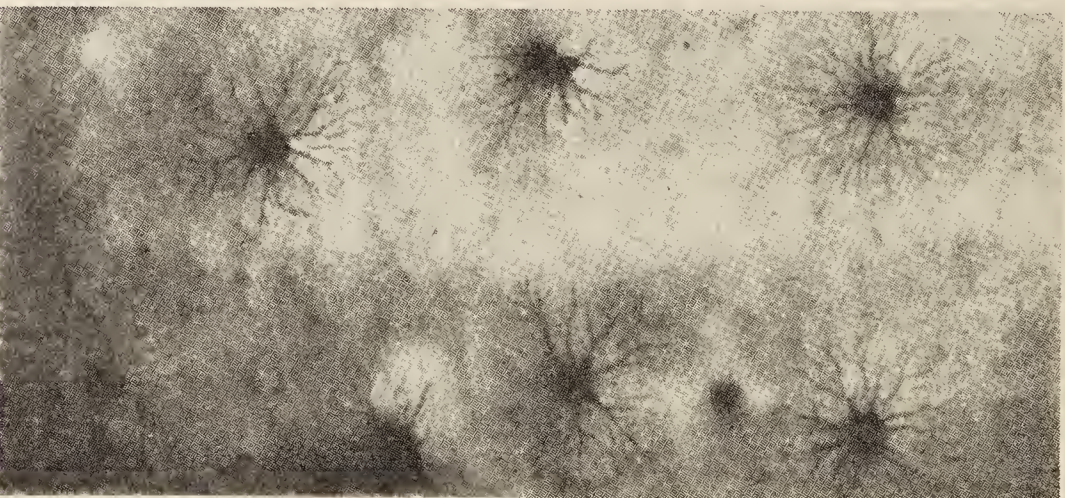
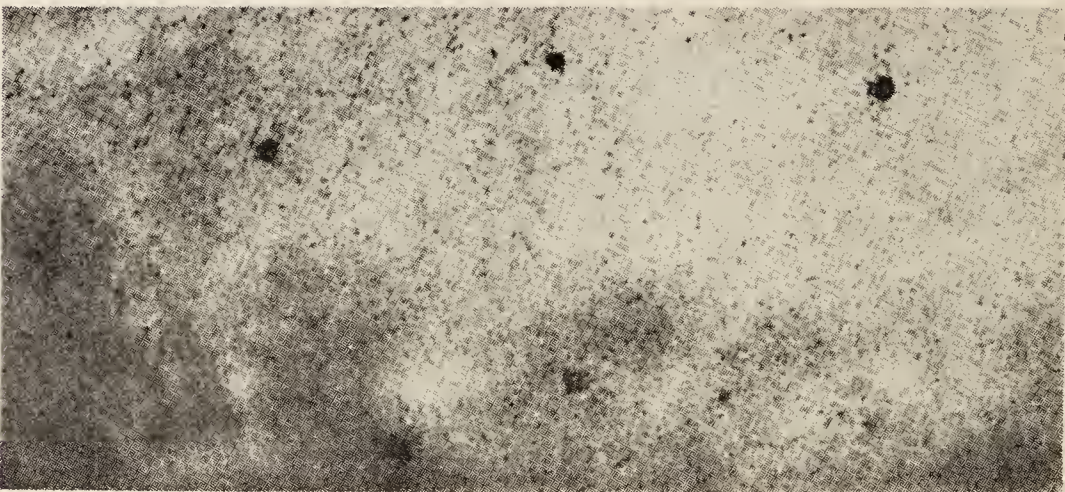
The most obvious conclusion—one that has been supported by all the experiments—was that the fruiting bodies give off a gas and that they orient in a gradient of the gas, leaning away from regions of high concentration. Our proof, however, still remains somewhat indirect, because the fruiting bodies are so minute and because it is not practicable to sample and assay a gas gradient of such tiny dimensions.

We did get a measure of the sensitivity of the mechanism. When the cell masses are placed more than .8 millimeter apart, they grow straight up in the air; at less than this distance they repel one another. The shorter the distance between them, the greater the mutual repulsion. With the cell masses side by side and touching, the two stalks rise at an angle of 45 degrees to the surface and make a perfect 90-degree angle to each other.

Similarly, we found that the stalks will lean away from the surfaces of inert objects, such as agar blocks, when they are placed close enough to allow a significant concentration of gas to develop in the space between stalk and surface. A growing stalk will even lean away from a glass rod planted next to it in the agar culture medium. With thin glass cover slips we were able to demonstrate geometrical precision in the response: the stalks always grow or lean equidistantly from the confining walls.

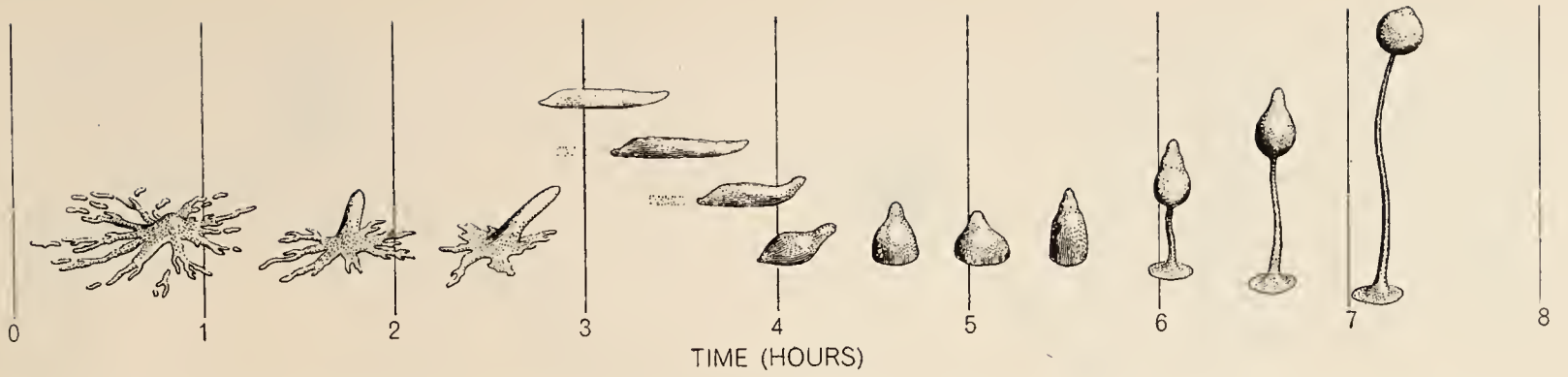
If a gas is given off, it should be possible to blow it away. With the help of D. C. Hazen of the Princeton aeronautical engineering department we constructed the world's smallest wind tunnel, with an aquarium pump for the source of its wind. Exposed to a breeze of 30 to 200 centimeters per second, solitary cell masses invariably gave rise to fruiting bodies that leaned into the wind. This was strong support for the gas hypothesis. Because the wind blew the gas to the leeward of the slime mold, the fruiting body would tend to grow "upwind" and away from the gas.

Given the tiny dimensions of the fruiting body and therefore of the gas gradi-



**AGGREGATION** of social amoebae is the first stage in the formation of slime-mold fruiting bodies. In the photograph at the top thousands of amoebae are spread evenly over an agar surface; an unknown substance repels them from one another as they forage for bacteria. In the second picture the food has been depleted and the amoebae begin to stream toward central collection points, attracted by a substance called acrasin, which is secreted by a few "founder cells." In the third and fourth pictures most of the amoebae in the area have become concentrated into central cell masses. The even spacing of the aggregates is accomplished by means of an aggregation-inhibiting gas, also given off by the founder cells.





**LIFE CYCLE OF A SLIME MOLD**, typified in this series of drawings by the species *Dictyostelium discoideum*, covers the period from aggregation (*far left*) to the development of a mature fruiting

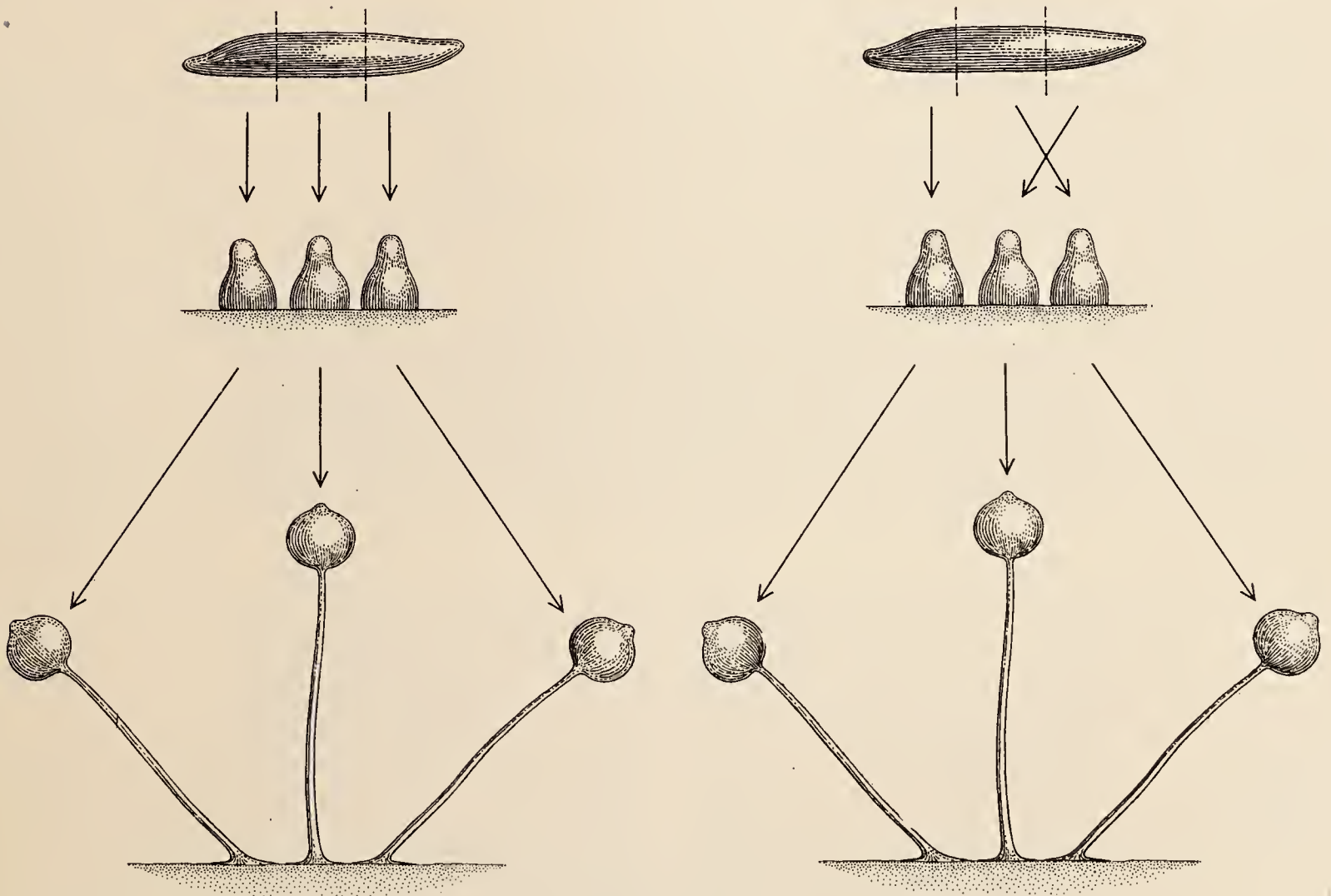
body (*far right*). Between these two stages the cell mass migrates about for a time in the form of a slimy, sausage-shaped slug before settling into its final fruiting position. Times are only approximate.

ent, one could predict that in a very small chamber the total concentration of the gas would rise to such a level that the gradient would effectively disappear. A small piece of glass tubing, three millimeters in diameter, was heat-sealed at one end and the other was plugged with agar containing one or two cell masses. Culmination took place even though the volume of the chamber was only about

20 cubic millimeters. But the fruiting bodies showed a total lack of orientation; the stalks grew neither straight nor upward, nor did they avoid each other or the glass or the agar surface. With mineral oil, a strong absorber of gases, we found that we could produce the same effect but for exactly opposite physical reasons. Submerged in the oil, the cell masses threw up fruiting bodies,

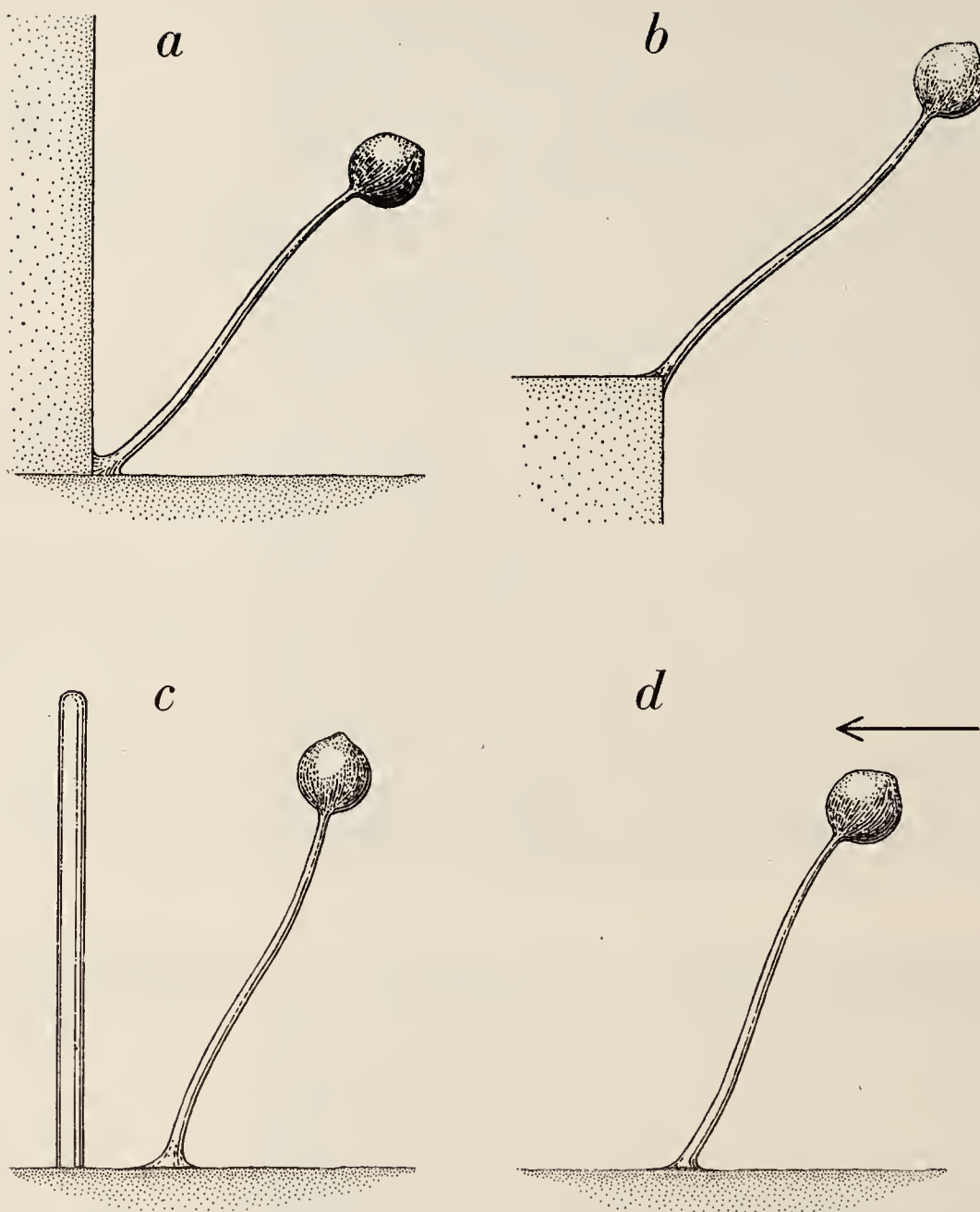
but in a completely disoriented fashion. In this case the gas phase was totally eliminated by the oil.

The best evidence for the gas hypothesis came from an experiment in which we placed a cube of charcoal alongside the cell mass. Instead of being repelled by this mass of material, the fruiting body culminated right into the charcoal. Presumably the charcoal absorbs the gas



**CRUCIAL EXPERIMENT** that indicated the operation of a gas mechanism in the orientation of slime mold fruiting bodies was performed by two senior students at Princeton University, J. Rorke and G. Rosenthal. The drawing at top left shows a migrating slug cut into three pieces; when these fragments culminate (*bottom*

*left*), they tend to lean away from each other. If two of the fragments are exchanged (*top right*), the identical effect ensues (*bottom right*). This experiment showed that orientation has nothing to do with what part of the slug the cells come from; the only significant factor is their relation to each other as they rise.



FOUR DEMONSTRATIONS of the sensitivity of a cellular slime mold's gas-orientation mechanism are depicted. A cell mass placed at the base of an agar cliff (*a*) tends to bisect the angle of intersection of the two surfaces. A cell mass placed at the crest of the cliff (*b*) culminates at an angle of about 135 degrees from each surface. When a small glass rod is planted in the agar near a rising fruiting body (*c*), the stalk leans away slightly from the rod. In a wind tunnel (*d*) the orienting gas is blown to the leeward of the fruiting body, causing the stalk to lean away from the gas and into the wind. In all four of these cases the fruiting body tries to maintain an equal concentration of gas on all sides.

and as a result there is less gas on the charcoal side. Subsequently B. M. Shaffer of the University of Cambridge showed that a drop of mineral oil would produce the same effect and would also cause migrating slugs to change course and approach the drop.

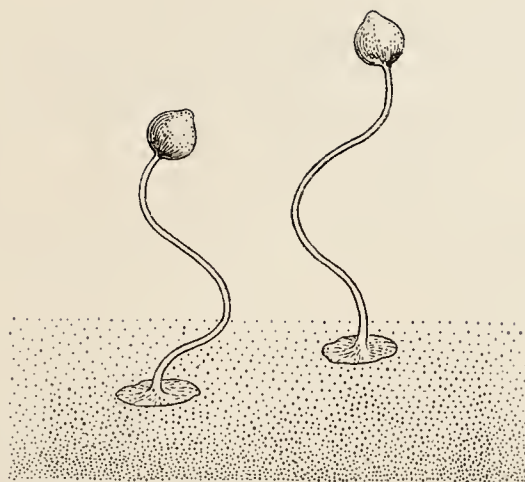
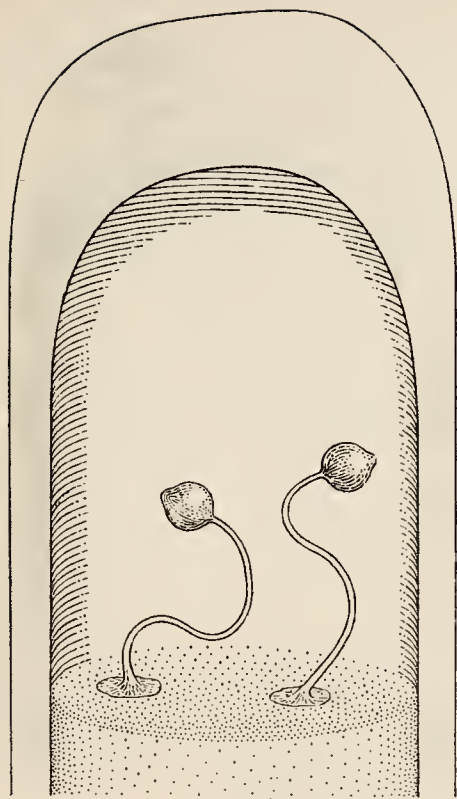
As for the chemical identity of the gas, we know at least that it is not specific to species; the gas given off by any species of slime mold will repel any other species. This suggests a common product of metabolism, such as carbon dioxide or ammonia. As will be seen, however, the question remains unsettled.

Whatever the nature of the gas, it is apparent that a vertical concentration

gradient of the gas, under natural and normal circumstances, causes the fruiting body of the slime mold to grow straight up at right angles to the surface. The next question is: What good does this do the slime mold? There is no certain answer because we do not even know the evolutionary significance of the aggregation of the amoebae into cell masses in the first place.

For the moment let us grant that rearing the fruiting body into the air is advantageous and further assume that it serves the protection and dispersal of the spores. In nature, fruiting occurs in the small caverns and chambers that riddle the humus and upper crust of the





**LACK OF ORIENTATION** of slime mold fruiting bodies was achieved in two different ways. A thin piece of glass tubing was heat-sealed at one end to form a tiny chamber (*left*) in which the concentration of gas rose so high that the fruiting bodies inside were unable to establish an effective orientation gradient. When mineral oil was poured over some cell masses (*right*), they also culminated successfully, but in an entirely disoriented fashion.

soil. If it is advantageous to store the spores free from any surface, obviously the gas orientation will be extremely useful: it will keep the spore mass in the center of any cavity. It may be that dispersal is primarily effected by worms and grubs slithering by and touching the ball of spores. As soon as an object touches the spores, they come off and stick to the foreign object by surface tension. Finally, if a number of fruiting bodies should grow close together in a large pocket in the soil, the gas-orientation mechanism will space the rising spore masses so that they spread over the whole area in an optimal way, filling

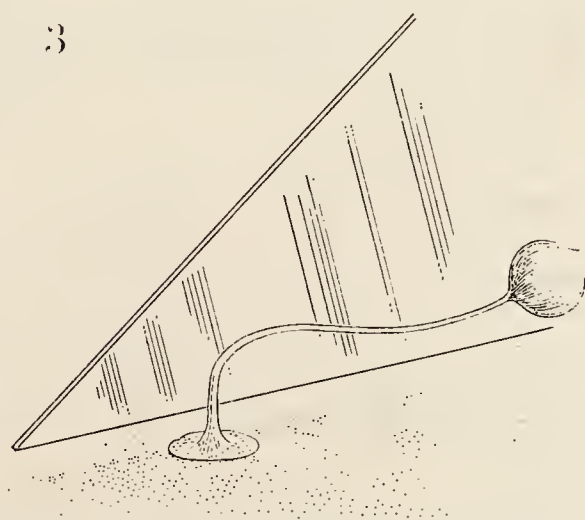
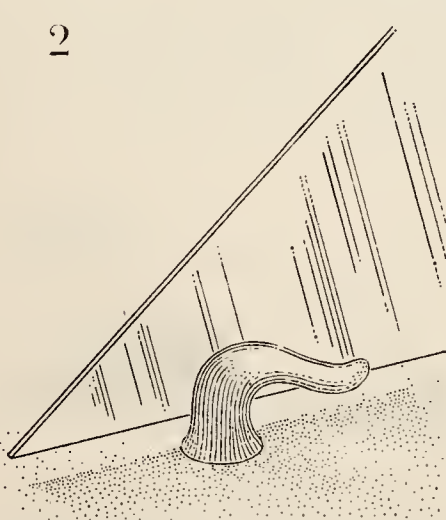
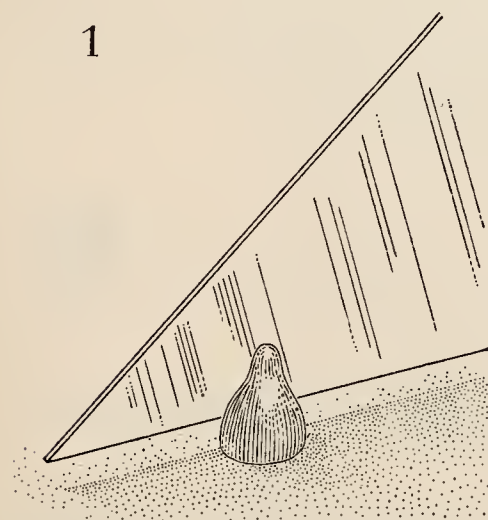
all the available space. The mechanism works with such precision that it is perhaps not unreasonable to expect that the growth of many other primitive plants—for example, simple fungi—is also oriented by gas gradients.

Meanwhile we had been working on what we thought was an entirely separate problem. In 1937 the German biologist A. Arndt observed that the number of fruiting bodies that formed in a culture dish appeared to be independent of the total population of amoebae in the culture. We confirmed this observation in experiments with five

different species. To put the story the other way around, it appeared that the size of the territory in which an aggregation of amoebae forms is constant (under given experimental conditions). Roughly speaking, if there are 10 amoebae in the territory, they will aggregate to produce one fruiting body of 10 cells, and if there are 10,000 amoebae, the 10,000 will join in a single, much larger aggregation.

All of this was of interest as a lead to the mechanism that initiates aggregation. Such evidence was inconsistent with the “initiator cell” hypothesis of Maurice Sussman of Brandeis University. He had proposed that one cell in every 300 or 2,000 (depending on the species) was somehow capable of starting aggregation. The finding that aggregation is a function of space rather than cell density fitted in more readily with an observation by Shaffer; he demonstrated that a single cell, which he calls the founder cell, is the focal point of aggregation and that this cell inhibits other founders from forming in its immediate vicinity.

In the fall of 1962 we performed a simple experiment that immediately suggested the nature of the inhibitory influence exerted by the founder cell. We placed amoebae of the species *Dictyostelium mucoroides* in tightly stoppered culture tubes. No aggregation took place; this was not surprising because it had been shown by James H. Gregg of the University of Florida that the cells require oxygen for aggregation and the later stages of development. In one of these tightly stoppered tubes, however, added a spatulaful of activated charcoal. Within an hour aggregation was fully under way in this tube. More careful repetitions of this experiment showed that it



**ELEGANT CURVE** in the stalk of a rising fruiting body can be produced by placing a microscope cover glass over the cell mass

during culmination. The slime mold’s gas-orientation mechanism tends to keep it equidistant from both the cover glass and the agar.



was; not the absence of oxygen but the accumulation of some inhibiting gas that prevented aggregation in the closed tubes. We found also that aggregation would occur if we reduced the number of amoebae per culture tube below a certain threshold. From this it could be calculated that the presence of more than approximately 250 cells per cubic millimeter of air space would inhibit aggregation. Apparently if the cells were fewer, the gas did not accumulate in sufficient concentration to prevent aggregation. At threshold concentrations the amoebae formed abnormal aggregations or disoriented fruiting bodies. All these peculiarities disappeared when we added a small heap of charcoal to the culture.

From the known size of the chamber and the concentration of the amoebae it was possible to compare territory size or, conversely, to compute the number of fruiting bodies per square centimeter in the presence or absence of charcoal. It turned out that the fruiting bodies were approximately four times denser or more crowded together in the presence of charcoal. In other words, removal of the inhibiting gas reduced the size of the aggregation territory. This conclusion was dramatically fortified when we submerged the cultures in mineral oil—the territory size then became minute. In this case, however, it is not certain that the effect is exclusively due to the removal of the inhibitor. Raper showed some years ago, for instance, that drying markedly reduces territory size; oil might somehow promote this effect.

On the precedent of our wind-tunnel experiments in the orientation of the growth of fruiting bodies, we compared the sizes of territories formed in still and in circulating air. In circulating air the density of the fruiting bodies was four to nine times greater than in still air. Therefore by disturbing the normal diffusion pattern of the gas and blowing it away as well one can effectively reduce the territory size.

Other experiments showed that the gaseous "spacing substance" is not species-specific: each one of four different species cultured in the same confined chamber with *Dictyostelium mucoroides* produced a gas that inhibited the formation of centers of aggregation and correspondingly increased the territory size of the *Dictyostelium mucoroides* fruiting bodies.

Turning to the task of identifying the spacing substance chemically, we found that carbon dioxide is the one

gas that will produce the same effect. On the one hand, the addition of approximately 5 per cent carbon dioxide to the atmosphere of the culture tube totally inhibits aggregation in *Dictyostelium mucoroides*. On the other hand, substances that absorb  $\text{CO}_2$  selectively will lift the inhibition, although not so effectively as charcoal. The gas chromatograph showed no additional spikes other than the ones common to room air in samples of gas taken from tubes in which cultures were inhibited.  $\text{CO}_2$  is, of course, one of the gases common to room air.

Can we conclude that the spacing substance is  $\text{CO}_2$ ? It would be most unwise to do so at this time. The gas may merely imitate the natural spacing substances, one of which may be  $\text{CO}_2$ . I have a particularly strong reason for caution. We made all these tests with five species and found only two of the five sensitive to the gas, although they all produced it. Two species are totally insensitive at all times to either naturally produced spacing substance or added  $\text{CO}_2$ . Most mystifying of all, the fifth species sometimes exhibits sensitivity to the gas and at other times appears to be totally insensitive. It seems to be able to switch its sensitivity on and off. Much more work must be done before we can say we have identified the spacing substance.

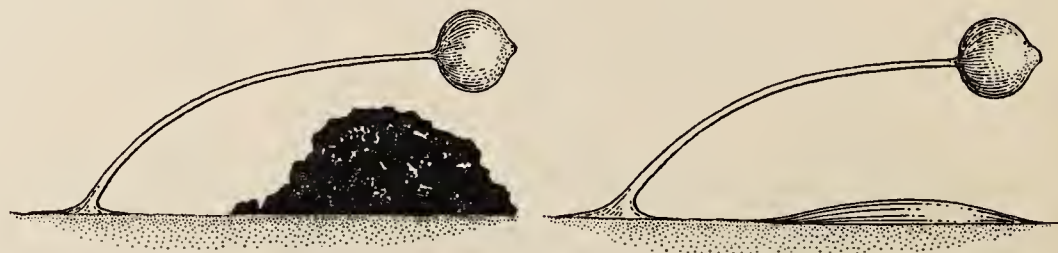
In the case of *Dictyostelium mucoroides*, one of the species sensitive to the gas, we were able to demonstrate that it produces the gas not only at the initiation of the process of aggregation but also throughout the entire process and during migration and culmination as well. Thus when the fruiting body of a large cell mass rises above a culture swarming with amoebae in the preaggregation phase, the evolving gas totally inhibits aggregation in the rest of the culture until fruiting is completed. This suggests that in nature aggregation may occur in waves, each one starting only after all the fruiting of the previous wave is complete. We found also that, if conditions allowed a high accumulation of the spacing substance, migration is cur-

tailed. Barbara Wescott, working in Raper's laboratory, had shown previously that a concentration of 5 to 10 per cent  $\text{CO}_2$  in the atmosphere of the culture inhibits migration.

The fact that the gaseous spacing substance is produced during migration and culmination strongly indicates that this gas and the gas that orients the growth of the fruiting body are one and the same. Neither gas shows species-specificity and both are adsorbed by charcoal and mineral oil. What is more, from the point of view of their function they both do the same thing. At aggregation the substance controls the spacing of the aggregates; as the cell masses rise from the surface, they are again "spaced" by the mechanism of gas orientation. In the first case the spacing is in two dimensions; in the process of orientation the spacing effect is three-dimensional. Certainly we are justified in calling both gases spacing substances.

It is now permissible also to speculate about how the spacing substance produces its effects. One hypothesis that simultaneously accounts for the inhibition of aggregation and for orientation of the fruiting body suggests itself: the spacing substance tends to speed up the movement of the cells. Centers of aggregation are formed by a localized slowing of cells; if the cells are speeded up, they are inhibited from forming centers. Similarly, if two fruiting bodies are close together, the gas concentration will be highest between them; the cells on this inward side will then move more rapidly, and the growing stalks will bend away from each other. This hypothesis is attractive, but it remains to be tested.

What is established is that the multicellular cell masses of the slime mold can converse with one another by means of a gas. We have always known that the social existence of many higher animals is influenced by odors. Now we have to learn more about the gas communications of slime molds and of other microorganisms as well.



ADSORBING THE ORIENTATION GAS by a piece of charcoal (left) or a drop of mineral oil (right) lowers the concentration of gas on the side of the adsorbent, causing the stalk to lean in that direction. Migrating slugs can also be attracted toward these adsorbents.

## THE ACRASIN ACTIVITY OF ADENOSINE-3',5'-CYCLIC PHOSPHATE\*

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*Communicated by Colin S. Pittendrigh, July 27, 1967*

The life cycle of the cellular slime mold is marked by a phase of vegetative reproduction and, after the exhaustion of the food supply, a phase of morphogenetic activity. The first visible event to occur after the onset of starvation is the aggregation of the myxamoebae into a mound of cells which subsequently differentiates into a migrating slug and ultimately into a fruiting body of varying complexity. It has been recognized for some time that this first event, the aggregation of cells, is mediated by a chemical messenger which attracts the cells over a distance, presumably by establishing a diffusion gradient against which these cells may orient.<sup>1</sup> The chemical itself has been named acrasin.<sup>1</sup>

Attempts to identify this substance have been reported by Shaffer,<sup>2</sup> Sussman,<sup>3</sup> and Wright,<sup>4</sup> all using the Shaffer assay.<sup>5</sup>

Konijn<sup>6</sup> reported that *E. coli* is able to attract myxamoebae from a distance, and Bonner<sup>7</sup> has suggested that this material may be chemically identical to acrasin. This may indeed be so since amoebae are most sensitive to this attraction just prior to aggregation.<sup>8</sup>

We wish to report that purification of this material, using a different assay,<sup>9, 10</sup> has now progressed sufficiently far to be able to make significant comments upon its chemical structure. We suggest that the attracting compound may very likely be adenosine-3',5'-cyclic phosphate (3',5'-cyclic AMP), and that pure, crystalline material obtained from a commercial source shows extremely high activity in the bioassay.

*Materials and Methods.—Bioassay:* Details of purification and bioassay will be published subsequently, though some information on the assay is already available.<sup>9, 10</sup> Droplets of a suspension of myxamoebae were deposited on hydrophobic agar of defined rigidity such that all cells remained within the margin of the drop. The test solution (0.1  $\mu$ l) was placed nearby (100–500  $\mu$ ), and the assay was scored positive if cells moved outside the boundaries of the droplet.

*Purification:* Purification involved repeated gel-filtration on Sephadex G-10, followed by descending paper chromatography using a solvent system consisting of butanol, acetic acid, water (4:1:5). Finally, the active fraction was run on paper electrophoresis with pyridine and acetate buffer at pH 3.9.

*Spectra:* Ultraviolet spectra of our purest fraction were obtained on a Cary, model 14 recording spectrophotometer.

*Chemicals:* 3',5'-Cyclic AMP and 5'-AMP were obtained from Calbiochem, and Mann Research Biochemicals, respectively. They were of the highest available purity. Sephadex G-10 was obtained from Pharmacia Fine Chemicals.

*Strains:* *E. coli* B/r and *D. discoideum* NC-4(H) were used in these experiments.

*Results.—*Attracting activity was isolated from several strains of *E. coli* and other bacteria. Activity was also found in human urine from both male and female.



TABLE 1  
DATA FOR ACTIVE FRACTION AT PRESENT PURITY

Property	Result
Molecular weight by gel-filtration	200-400
Net molecular charge in electrophoresis at pH 3.9	Minus
$\epsilon_{260}$ at pH 10.5	270
$\lambda_{\max}$	259 m $\mu$
Heat stability	High

To date, the following information is available concerning the active component of the *E. coli* extract. Gel-filtration on Sephadex G-10 indicates a molecular weight of between 200 and 400. It is heat stable. Migration of the active band in paper electrophoresis indicates a negative charge for the molecule, even at low pH. Ultraviolet absorption spectroscopy shows a prominent, symmetrical peak at 259 m $\mu$  at pH 7. A shift in the shape of the curve is seen at pH 2, although the  $\lambda_{\max}$  remains the same. The spectrum at pH 12 is identical to that at pH 7. These data suggest that the absorbing material might be an adenine derivative. The extinction coefficient at 260 m $\mu$  ( $\epsilon_{260}$ ) is 270. This indicates that about 2 per cent of our purest material absorbs ultraviolet if it has an  $\epsilon_{260}$  of 14,200.<sup>11</sup>

Even at this low purity, however, we felt that the data were sufficiently indicative to warrant investigation of several known adenine nucleotides. 5'-AMP gives no activity. 3',5'-Cyclic AMP has shown activity in our assay at amounts as low as 0.01 m $\mu$ g. Amounts as low as 2.50 m $\mu$ g of our purest fraction give similar activity. An estimation of purity from the bioassay is therefore in the same order of magnitude as that from the spectrophotometric data.

*Discussion.*—It seems to us likely that 3',5'-cyclic AMP is responsible for the activity from *E. coli*. It is known that *E. coli* produces this material in large quantities.<sup>12</sup> It would be isolated by our procedure, and our assay would detect it. We do not, of course, know whether there are other compounds with similar properties.

It is also interesting to note that we have obtained activity from human urine, and that preliminary data show it to migrate similarly to *E. coli* activity on Sephadex G-10. Human urine contains significant quantities of 3',5'-cyclic AMP.<sup>12</sup>

We recognize that the attraction of myxamoebae towards an extract of *E. coli* does not identify 3',5'-cyclic AMP as the chemical messenger which mediates the normal morphogenesis of cellular slime molds, although we consider this likely. We are especially intrigued with the fact that sensitivity to the *E. coli* extract is highest just before aggregation of the myxamoebae. Specificity of aggregation in mixed populations of cells may reside in a mechanism such as differential adhesion of cell membranes, rather than in the messenger itself.

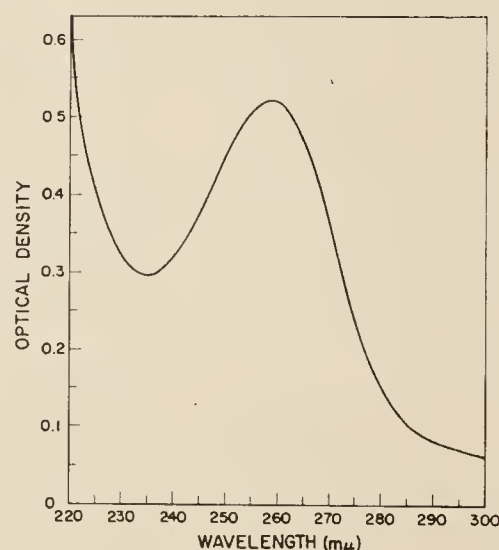


FIG. 1.—Ultraviolet absorption spectrum of purest fraction derived from *E. coli* extract.

Experiments designed to identify the active fraction of the *E. coli* extract, and the active material in normal slime mold morphogenesis are underway.

*Summary.*—3',5'-Cyclic AMP has been found to attract sensitive slime mold myxamoebae. It is active in amounts of 0.01 mμg. It is suggested that this is probably the orientation factor in *E. coli* responsible for the attraction of sensitive myxamoebae. The implications of this finding with reference to slime mold morphogenesis are discussed briefly.

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## CYCLIC AMP: A NATURALLY OCCURRING ACRASIN IN THE CELLULAR SLIME MOLDS\*

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One of the key events in the morphogenesis of the cellular slime molds is aggregation. The separate amoebae stream together to form a multicellular organism that undergoes morphogenetic movement and differentiates as a unit. The ultimate result is a fruiting body consisting of a delicate stalk and an apical mass of spores.

Because it has been described so often elsewhere (e.g., Shaffer, 1962; Bonner, 1967), this is not the place to give a detailed history of the evidence that aggregation is mediated by chemotaxis. Suffice it to say that the first positive indication that this might be the case was the demonstration of Runyon (1942) that centers can orient amoebae even though the centers and the amoebae are separated by a semipermeable membrane. That this could be explained on the basis of a diffusion of a small molecule was confirmed and firmly established by Bonner (1947), who called the amoeba-attracting substance *acrasin*. Shaffer (1953) was the first to isolate the substance in vitro, reintroduce it, and obtain the orientation of sensitive amoebae. He also showed that the substance was destroyed, probably by an enzyme (Shaffer, 1956*b*), a point which was confirmed by Sussman, Lee, and Kerr (1956).

Since 1956 there has been an active search for the chemical identity of acrasin in a number of laboratories. This was possible because Shaffer (1953, 1956*a*) devised a chemotaxis assay which consists of sandwiching amoebae (which are at a sensitive stage) between a small agar block and a glass slide. The test solutions are added to the edge of the agar block at appropriate time intervals, and if the solution contains acrasin the amoebas under the block will stream toward the edge. Shaffer's test was one of the major advances in the study of aggregation; yet, mainly because

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Much of the work described in this paper is new, and therefore this is a preliminary announcement. The material is also being prepared for detailed publication. This work was done on a NATO-Science Fellowship administered by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.)

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it was not quantitative, it has been disappointing as a practical help to the biochemist who seeks the molecular nature of acrasin.

The result has been a series of contradictory and puzzling results from various laboratories (review by Bonner, 1967). Steroids, alkaloids, and other less clearly defined substances were reputed to have shown acrasin activity, but most workers felt that the experimental evidence in all these cases was unsatisfactory and that the matter was still open and unsolved.

To overcome these difficulties a quantitative assay was developed independently by Konijn and Bonner. Last spring we decided to pool our efforts and work for a period in the same laboratory. It has become clear that Bonner's test, which involved the effect of the extracts on the rate of movement of amoebae, is not satisfactory for this purpose, although it did help us to move in what turned out to have been the right direction (Bonner, Kelso, and Gillmor, 1966). On the other hand, Konijn's test has proved to be satisfactory in all respects. Because it is so basic to the further work to be described here, it is important to stress its major features.

There are two essential components to Konijn's test: one is a method of preparing the agar so the responding amoebae are suitably constrained, and the other is a method of insuring that the amoebae are all at an acrasin-sensitive stage at the same time.

The agar must be hydrophobic so that the test cells remain trapped in the area of the initial drop. This is achieved by repeated washing of the agar with distilled water. The agar must also have the right hardness so that those amoebae which are attracted by acrasin will break loose from the confines of the initial drop and escape below the surface into the slightly soft agar gel. This latter property is simply achieved by carefully controlling the concentration of the agar. To avoid the deleterious effects of hypotonicity, Bonner's salts are added to the agar (Konijn, 1965; Konijn and Raper, 1966).

The test consists of spotting a series of drops of amoebae on such an agar surface and placing a drop of test solution near each circle filled with amoebae. If the solution has acrasin activity, the amoebae will burst from the confines of the circle and penetrate into the agar. The assay can be made quantitative by doing a number of tests with different distances between the solution and the amoebae drops to find the distance at which 50% of the cases show attraction.

However, for effective testing, it is essential to have the amoebae at just the right stage. This is achieved by harvesting amoebae that have not yet begun to aggregate from growth plates and placing them in the drops on the special agar. These plates are then kept at 22° C in the dark for about 3-4 hr followed by 12 or more hr at 5° C in a refrigerator. When they are taken back to room temperature they will immediately be receptive to acrasin, and furthermore the response appears to be synchronized for all the amoebae. This procedure works for various species, but it appears especially effective for *Dictyostelium discoideum*, the species that Shaffer (1953) previously showed was the most satisfactory for assay purposes.



This temperature-delay method of synchronizing the sensitivity of cells is such an important element in the test that we have been able to demonstrate at Princeton that attraction to acrasin solutions is possible without the special agar. If drops of *D. discoideum* amoebae are placed on unwashed 2% Bacto agar, the cells will immediately spread from the original circle. Nevertheless, if, after the period of 5° C, an agar block containing acrasin is placed at the edge of the circle, the cells will stream toward the block (Fig. 1, *top*). We have called this modification of Konijn's test the agar

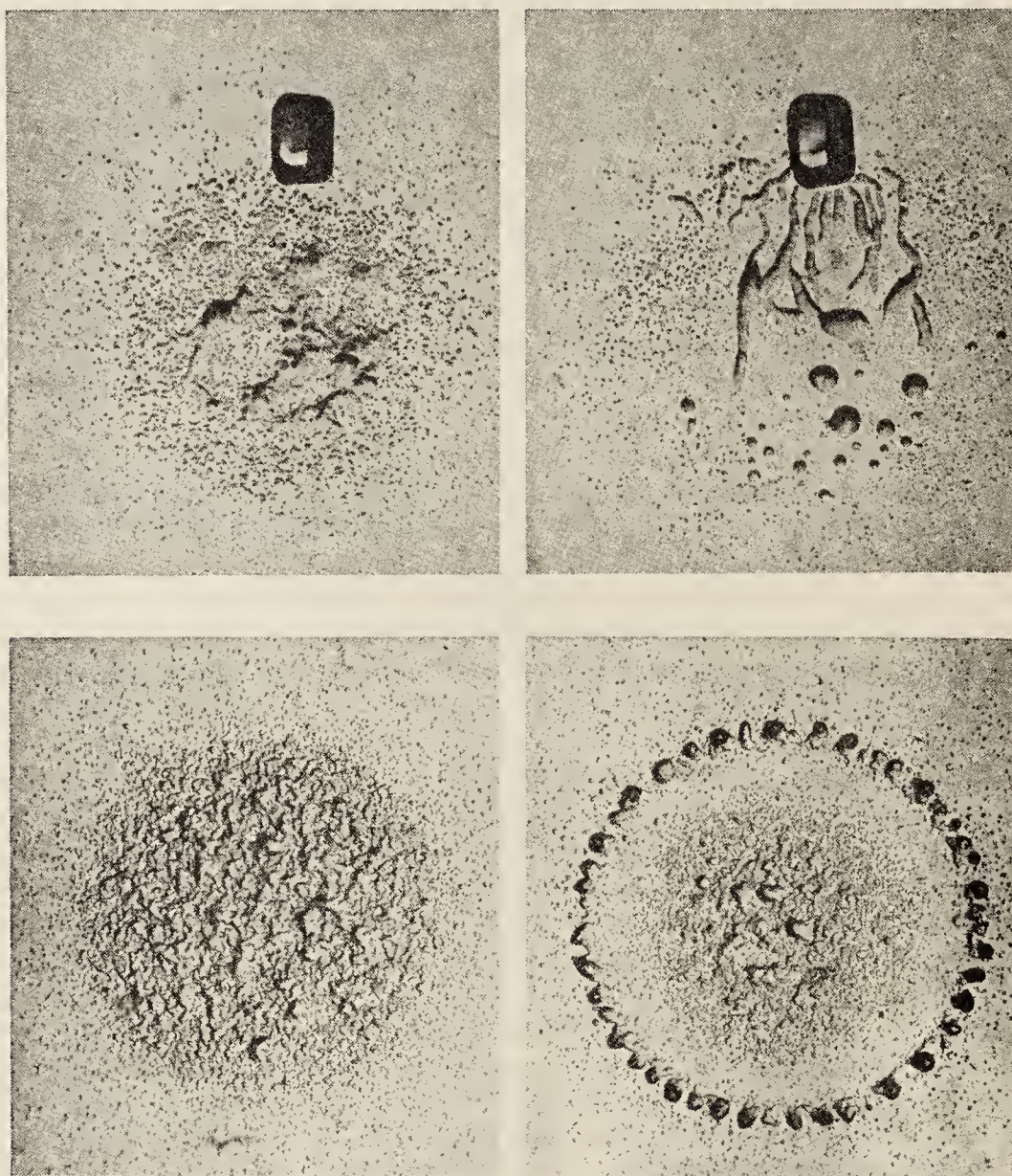


FIG. 1.—*Top*: The agar block test. *Left*: An agar block containing .05 mg/milliliter of cyclic AMP has been placed near a drop of amoebae that are just beginning to aggregate. *Right*: 57 min later. *Bottom*: Ring formation caused by cyclic AMP in the agar. *Left*: Control with no cyclic AMP in the agar, after 7 hr. *Right*: A similar drop of amoebas after being in agar containing 0.1 mg/milliliter of cyclic AMP.

block test. It can be made quantitative by scoring the percentage of the amoebae that enter the block, but it is far less sensitive than the original test.

#### DEMONSTRATION THAT CYCLIC AMP ACTS AS AN ACRASIN

Some years ago, using his test, Konijn (1961) showed that bacteria (*Escherichia coli*) would attract slime mold amoebae, a fact independently shown by Samuel (1961) using less refined methods. More recently Konijn showed that the supernatant from bacteria, prepared by centrifugation, was also active in his test. This was confirmed in the Princeton laboratory both on the rate test (Bonner, Kelso, and Gillmor, 1966) and on the agar block test. Bonner also was able to show that human urine gave high activity on both these tests.

The most successful efforts on the chemical purification of these extracts were made by Konijn and his co-worker in Utrecht, J. G. C. van de Meene. They were able to show that the active component in the extracts had a low molecular weight somewhere between 200 and 400, a negative charge, that it was heat stable, and had a high absorption peak at 259 m $\mu$ . It was Barkley who suggested that the compound might be cyclic 3', 5'-adenosine monophosphate, a compound known from the work of Sutherland and his co-workers to be present in *E. coli* and human urine (review by Sutherland, Øye, and Butcher, 1965). Konijn was able to show immediately that this compound had an extraordinary capacity to attract amoebae. It is possible to produce a positive result in the assay with amounts as low as .01 m $\mu$ g of cyclic AMP (using drops of 0.1  $\mu$ l). Once this discovery was made, there was little difficulty in showing that the only active component of the bacterial supernatant was cyclic AMP (Konijn, van de Meene, Bonner, and Barkley, 1967, and in preparation).

The orienting effect of cyclic AMP is best illustrated in *Dictyostelium discoideum*, as is shown in Figure 1, top. Clearly, *D. mucoroides* and *D. purpureum* are also attracted by this substance. Its effect on *Polysphondylium pallidum* and *P. violaceum* is puzzling and unresolved. This ties into the whole problem of species specificity of acrasin first examined by Shaffer (1953, 1957a), and at the moment we are looking into the matter of whether there are other acrasins besides cyclic AMP.

#### DEMONSTRATION THAT CYCLIC AMP IS A NATURALLY OCCURRING ACRASIN

The key question is whether cyclic AMP is synthesized by cellular slime mold amoebae. By chance we first attacked this problem with *Polysphondylium pallidum* grown on dead (autoclaved) *Escherichia coli*. The original growth medium containing the killed bacteria had a small residual attraction activity on Konijn's test, but after the *P. pallidum* had consumed the food, the supernatant from the amoebae showed a great increase in acrasin activity in the assay. The increase in activity must have been caused by the amoebae, since there were no live bacteria present.

In order to find out if this amoeba-produced attractant is cyclic AMP, the



amoeba supernatant was concentrated and put on a Sephadex G-10 column. The active fractions were pooled and run through a DEAE Sephadex column, and again the active fractions were isolated. They were then mixed with activated charcoal and the eluate was put on paper and developed with three different solvent systems. In all three cases the  $R_f$  of the attractant corresponded exactly to that of purified, commercial cyclic AMP. We also treated the purified active material from the amoebae with the specific enzyme phosphodiesterase, obtained from beef heart (kindly sent to us by R. W. Butcher), and found that it destroyed the ability to attract. These two approaches leave little doubt that *P. pallidum* synthesizes cyclic AMP.

We are in the process of attempting a similar demonstration with *Dictyostelium discoideum*. Although there is clear evidence that *D. discoideum* synthesizes an attractant, initial difficulties have been encountered in obtaining enough quantities of the substance to make further chemical purification. The apparent reason for these difficulties has turned out to be a matter of considerable interest.

The evidence was that the attractant was often disappearing more rapidly than it was synthesized. Chang has been pursuing this problem, and she discovered that the amoebae of *D. discoideum* are pouring out considerable quantities of phosphodiesterase into the aqueous medium about them. When mixed with cyclic AMP, as a substrate, this enzyme hydrolyzes cyclic 3', 5'-AMP to 5'-AMP. In fact, it shows many similarities to the mammalian phosphodiesterase originally described by Butcher and Sutherland (1962). It is almost too obvious to add that our hypothesis is that this is the acrasin-destroying enzyme originally postulated by Shaffer (1956b).

#### OTHER EFFECTS OF CYCLIC AMP ON CELLULAR SLIME MOLD DEVELOPMENT

Besides the ability of cyclic AMP to attract amoebae, a number of other interesting effects have been observed:

In the first place, cyclic AMP increases the rate of movement of the amoebae. It does this at very low concentrations (5 m $\mu$ g/milliliter); however, it is clear that there are other, more effective rate-increasing compounds in bacterial extracts.

A more striking effect of cyclic AMP is the production of cell adhesiveness. This is a characteristic of acrasin first described by Shaffer (1957a, 1957b), and it can be effectively produced by simply including cyclic AMP in the agar on which sensitive pre-aggregation cells are placed. The cells will clump, sometimes forming small streams. It is interesting, in this connection, that ADP is known to cause adhesiveness in *Dictyostelium discoideum* as well as in other cells and in blood platelets (Jones and Wooley, cited in Born, 1967).

It has been shown by Bonner that the most effective way to demonstrate this phenomenon is to place drops of *Dictyostelium* amoebae on 2% Difco agar containing cyclic AMP. The amoebae will spread, but as they do so they will give rise to a tightly clumped ring of cells. In some cases, as the ring expands, it will break up into a ring of beads like a symmetrical crown

and each bead will wander out radially (Fig. 1, *bottom*). The hypothetical explanation of this orientation is that the cells deplete the concentration of cyclic AMP at the site of the drop by means of their phosphodiesterase and that this produces an ever widening circular gradient which orients the ring of amoebae in their outward expansion.

Another effect of cyclic AMP on *Dictyostelium* is center inhibition. Konijn observed some time ago that bacterial extracts inhibit center formation, and Shaffer (1966) independently reported this same fact. It is now possible to show the very same effect with cyclic AMP. For instance if a center is well advanced in a drop of *D. discoideum* cells and an agar block is placed at the edge of the drop, then the center will disintegrate and the cells will stream into the agar block containing the cyclic AMP (this can be seen in Fig. 1, *top*). The suggestion that center inhibition might in some cases be mediated by acrasin has been made by a number of workers, and now we have evidence that this is a genuine possibility.

The aggregation territory size of *Dictyostelium* becomes very much smaller with high concentrations of cyclic AMP. Why this should be so is not entirely clear; there are a number of possible explanations. Clearly, this is one more effect of cyclic AMP that must be examined in detail.

One final point should be mentioned concerning the effects of cyclic AMP. It is surprising that, even at high concentrations, this substance seems to have relatively little effect on differentiation. It is true that fruiting bodies on agar containing high concentrations of cyclic AMP may have a slightly abnormal appearance, due largely to a rough irregularity of the base of the stalk. At even higher concentrations all development will cease completely. Perhaps this lack of significant effect on differentiation merely reflects the fact that the substance is readily destroyed by the phosphodiesterase, especially when the cell mass has raised itself above the surface of the agar, and can isolate itself from the influx of cyclic AMP from the agar. In any event, there is no evidence thus far that cyclic AMP is an inductor in the sense of the animal embryologist.

#### DISCUSSION

With these preliminary facts before us, it is now time to give a more general assessment of where we are and where we are going. For 25 years Bonner has been concerned with the nature and the properties of acrasin, and it would almost seem as though relatively little progress has been made until the last few months. But is the knowledge of the chemical identity of at least one acrasin such an advance? Is it really an important stepping stone for the future?

There seems little doubt that this is so, just as it has been case for the identification of growth hormones in plants. Now that we have identified cyclic AMP, we can look for its biochemical control and try to correlate this control with the morphogenetic events which are so familiar to us. We already know of an enzyme which destroys this acrasin, and we are in a position (and even have some experiments under way) to examine other



aspects of this control. This is the kind of a biochemical approach to a developmental problem which would seem to have great possibilities for new insights.

None of this would seem so immediately possible were it not for all that is known about cyclic AMP. Since its isolation from mammalian tissue by Sutherland and Rall in 1958, there has been a great interest in its role in animals where it clearly is a substance of prime importance (reviews by Sutherland, Øye, and Butcher, 1965, and Smith, 1967). It is a so-called second messenger in many hormone reactions. The hormone in some way activates the adenylyl cyclase on the cell surface, which converts ATP into cyclic AMP and releases it inside the cell. Here the cyclic AMP triggers some other reaction, which results in the ultimate action of the hormone, and it does this before it is inactivated by a specific phosphodiesterase. The best known case which serves as an example of this type of effect is in the action of epinephrine. This hormone stimulates the adenylyl cyclase, and the resultant accumulation of cyclic AMP causes the nonactivated phosphorylase b kinase to be converted to its activated form. In this form it is capable of causing phosphorylase b to convert to phosphorylase a. This last compound is the one capable of converting glycogen into glucose-1-phosphate, the basic action of epinephrine in the mobilization of sugar in the body.

It is known that cyclic AMP is present in many tissues; it is affected by a large range of hormones and is capable of triggering an extraordinary number of internal reactions. The great importance of the substance in mammalian physiology is obvious; for us the more difficult problem is the relation of these specialized, complex hormone effects to chemotaxis in the cellular slime molds.

The fact that cyclic AMP is present in primitive systems was first shown by Makman and Sutherland (1965), who demonstrated that it was synthesized by *Escherichia coli*. But its function in bacteria is still obscure, although it is assumed that even in such lowly forms it is part of some biochemical regulating system.

At first it would appear that one difference between these lower organisms and mammals is that in the former the cyclic AMP seems to be mainly extracellular, while for mammals its actions are intracellular. Perhaps we are not yet in a position to make such a sweeping distinction; we do not know, for instance in the slime mold, what the cyclic AMP might be doing inside the cells, or for that matter if it goes into the cells during chemotaxis. Furthermore, in mammals high levels of cyclic AMP are found in the urine, and therefore it appears quite conceivable that it may play an important extracellular role in mammals also.

For instance, is it possible that it is responsible for certain cell aggregations in animals? These are known to occur both during development and in the adult. Is it conceivable that this might be one of the roles of this substance in vertebrates? Holtfreter (1968) has recently suggested parallels between slime mold aggregations and aggregations of chondroblasts, and indeed it would be interesting to pursue this parallel further.



To return to the cellular slime molds, if we compare them to mammals, it would appear that the cyclic AMP is for them the *primary* messenger rather than the secondary one. This would suggest that in bacteria the cyclic AMP is concerned with controlling some direct metabolic process; in cellular slime molds it has become a messenger as well and can carry the message across large intercellular spaces; in mammals a new messenger, the hormone, has been added which supersedes and relegates cyclic AMP to a second position or link in a chain. If one considers further the relation between various endocrine glands, then it is clear that the hormone chains themselves may be multiple, involving an even larger number of messages.

Such very general speculation is perhaps premature. There are many specific questions which can and in some cases are being examined at this moment. We must learn how cyclic AMP achieves its various morphogenetic effects, how it mediates chemotaxis, how it makes the cells more adhesive, and how it suppresses center formation. And furthermore we must learn how the cyclic AMP is itself controlled.

#### SUMMARY

There has been a long search for the chemical identity of acrasin, the chemotactic substance responsible for the aggregation of amoebae in the development of cellular slime molds. With the help of a new, quantitative assay, it was possible to show that supernatants from the bacterium *Escherichia coli* contained an attractant for the amoebae of *Dictyostelium discoideum*, and now it is known that this attractant is cyclic 3', 5'-AMP. Furthermore, we have evidence that cyclic AMP is synthesized by the related species *Polysphondylium pallidum*. It is also known that *D. discoideum* synthesizes an attractant, but as yet we have not been able to obtain large enough quantities for chemical analysis. This appears to be due to the secretion by the amoebae of large quantities of a phosphodiesterase that specifically breaks down the cyclic 3', 5'-AMP acrasin to 5'-AMP.

In mammals, from the work of Sutherland and his collaborators, it is known that cyclic AMP is a second messenger in hormone reactions. It is suggested that in the more primitive cellular slime molds, cyclic AMP serves as a primary messenger.

#### ACKNOWLEDGMENTS

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## Acrasin, Acrasinase, and the Sensitivity to Acrasin in *Dictyostelium discoideum*

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In the cellular slime mold *Dictyostelium discoideum* the separate amoebae feed on bacteria, and after the food supply is exhausted they aggregate to central collection points to produce small multicellular fruiting bodies consisting of an apical sorus supported by a delicate stalk. It has been known for some years that the aggregation process is mediated by a chemical substance, which was called acrasin (Runyon, 1942; Bonner, 1947; Shaffer, 1953), and that it was destroyed by an extracellular enzyme (which will be referred to as acrasinase; Shaffer, 1956a). Recently we have been able to show that adenosine-3',5'-cyclic phosphate attracts amoebae and therefore acts as an acrasin (Konijn *et al.*, 1967, 1968); that *D. discoideum* produces cyclic-AMP in substantial quantities (Konijn *et al.*, 1969a; Barkley *et al.*, 1969); and that this species also produces a phosphodiesterase that specifically converts cyclic-3',5'-AMP to 5'-AMP (Chang, 1968). There may be other unknown factors involved, but it is clear from this work that for *D. discoideum* cyclic-AMP is an acrasin and phosphodiesterase is an acrasinase.

These new facts raise the important and immediate question of how these two substances mediate the normal aggregation process. This paper is a contribution toward such an understanding. As a first step we have done experiments to determine when these substances are secreted during the life cycle of the organism, basic information necessary for an interpretation of their role in normal development.

The second approach has been to interfere with the external concentrations of acrasin and acrasinase in order to observe the effects upon the pattern of amoeba distribution. As we shall see, this has given some new insights into how the substances act upon the cells.

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## MATERIALS AND METHODS

These experiments were performed with *Dictyostelium discoideum*, haploid strain of NC-4. They were grown on *Escherichia coli*. The aggregateless mutants were obtained from irradiating spores of *D. discoideum* with ultraviolet light. The majority of these were isolated and kindly sent to us by Drs. R. A. Deering and J. Lennox of Pennsylvania State University.

*Acrasin determinations.* A special method was devised to separate the cyclic-AMP from the phosphodiesterase that destroys it. A dialysis membrane was stretched between two close-fitting plastic cylinders 8 cm in diameter (Fig. 1). The attached cylinders with the membrane between were placed in a crystallizing dish (5 × 10 cm), and the dish was filled with 1% physiological salt solution (Bonner, 1947) just up to the under-surface of the membrane. The amoebae were grown in sterile, liquid culture on dead, autoclaved bacteria [Hohl and Raper's (1963) modifica-

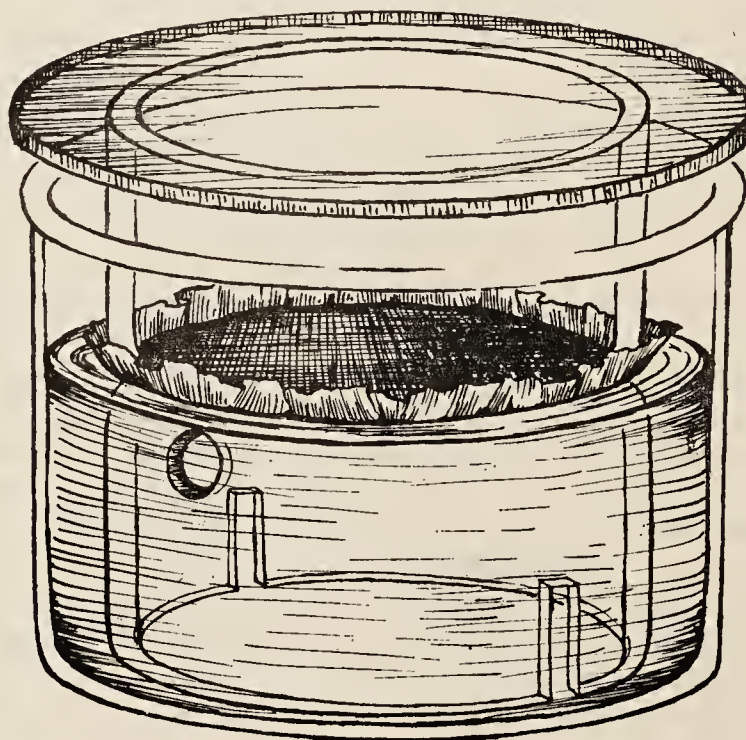


FIG. 1. A diagram of the dialysis tank to separate cyclic-3',5'-AMP synthesized by the developing amoebae from the phosphodiesterase that converts it to 5'-AMP. The amoebae are placed on the upper surface of the dialysis membrane, and the lower chamber is filled with 1% salt solution flush with the under side of the membrane. The lower solution can be removed at any time and analyzed for cyclic-AMP content.

tion of Gerisch (1959)]. Some hours before the bacteria were all consumed, the contents of the flasks were centrifuged, the amoebae were resuspended in 1 % salt solution and then added to the upper portion of the plastic chamber, so that the amoebae could settle on the surface of the membrane. As soon as they were attached to the cellophane the salt solution was siphoned off the upper chamber and the chamber was covered. In this way the amoebae were at an air-water interface on the upper surface of the membrane in a moist chamber, while below the membrane there was salt solution. Since only small molecules can penetrate the dialysis membrane, the cyclic-AMP secreted by the amoebae accumulated in the water below, separated from the destructive phosphodiesterase, which remained above the membrane.

The salt solution in the lower chamber was removed every 2 hours, concentrated first by boiling and then by vacuum evaporation at 60°C to dryness. It was resuspended in 0.1 ml of distilled water and tested on the Konijn test for acrasin (Konijn, 1965; Konijn and Raper, 1966). By simultaneously running a series of concentrations of commercial cyclic-AMP on the Konijn test, it was possible to compare the reaction of the test solution with that of known concentrations of cyclic-AMP. The range of concentrations used was very great and therefore this gives only a rough indication of the actual concentration of cyclic-AMP given off by the amoebae in the 2-hour interval.

*Acrasinase determinations.* Since Chang (1968) showed that the phosphodiesterase was secreted by the amoebae, the following simple method of determining the relative activity of the enzyme produced at different stages of the life cycle was used. The amoebae were grown in petri dishes on live bacteria on an agar medium of buffered 1 % peptone and 1 % dextrose. Some hours before aggregation had begun, the vegetative amoebae and the remaining bacteria were washed off the agar surface with 1 % salt solution, washed twice by centrifugation, and allowed to settle on HA Millipore filter paper which has a pore size sufficiently large to permit proteins to pass through readily.

Once the amoebae and bacteria had settled, the filter paper was placed over fresh 2 % nonnutrient agar for successive 2-hour periods. After such a 2-hour period, when the Millipore filter had been removed, a square of the agar (1 × 1 cm) was cut and placed in a moist chamber. Onto it was placed 0.1 ml containing 1  $\mu$ C of  $^3\text{H}$  cyclic-AMP (specific activity 2.35 C/mole from Schwartz BioResearch, Inc.). This was allowed to incubate for 30 minutes, after which the block was eluted with 3 ml of distilled water and immediately evaporated to dryness. Four

drops of water ( $\sim 0.05$  ml) were added, and the solution was placed on chromatographic paper. The chromatograph was run in a solvent system (1 *M* ammonium acetate-*ETOH*, 3:7) which is effective in separating cyclic-3',5'-AMP from its degradation product, 5'-AMP. Live bacteria alone were run as controls, and even though they are known to produce a phosphodiesterase (Brana and Chytil, 1966) it is entirely within the cell for they were unable to convert extracellular cyclic-AMP to 5'-AMP. Therefore all the enzyme activity recorded was the result of the phosphodiesterase secreted by the amoebae.

The chromatograms were cut up in  $1 \times 3.5$  cm strips and placed in Buhler's solution. By counting each portion of the chromatogram in a liquid scintillation counter, it was possible to identify the relative amounts of cyclic-3',5'-AMP and 5'-AMP, thereby indicating the relative activity of the slime mold phosphodiesterase for any particular sample.

## RESULTS

### *Acrasin Production during the Course of Development*

Once it had been demonstrated that the acrasin of *D. discoideum* was cyclic-AMP, the first question was whether or not it was produced at all stages of development.

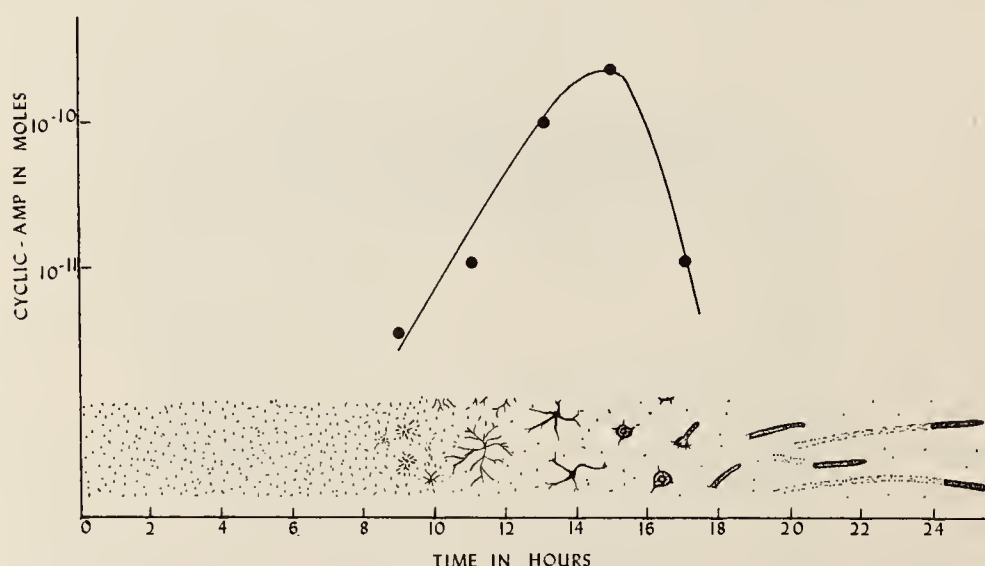


FIG. 2. A graph showing the results of one experiment in which the amount of cyclic-AMP given off for successive 2-hour intervals in the dialysis tank is plotted against time. The stage of development is indicated directly on the graph. (Note: The values for the concentration in moles are rough and approximate.)



A series of three experiments were run in which the water under the dialysis membrane in the chamber previously described was collected every 2 hours and analyzed for its cyclic-AMP content using the Konijn test. All the experiments showed the same result, but one of these extended over a particularly long time span and therefore is used as an illustration (Fig. 2). At the first signs of aggregation there is a sudden increase in cyclic-AMP production which reaches a peak in the latter part of aggregation stage, and then decreases. The overall increase is in the order of 100-fold.

It is not clear whether the vegetative amoebae are synthesizing some cyclic-AMP; at least it cannot be detected by the test. However, it is known from some old observations (Bonner, 1949) that the migrating slugs do produce some acrasin.

#### *Acrasinase Production during the Course of Development*

Two series of experiments were run to examine the activity of the phosphodiesterase during different stages of development. Again 2-hour periods were used which included the vegetative stage, the aggregation stage, and the migration stage. In each case the agar blocks were tested for their ability to convert 3',5'-AMP to 5'-AMP. It was possible to show that phosphodiesterase activity occurs throughout development (although the method was not sufficiently sensitive to obtain quantitative comparisons for the different stages).

#### *Sensitivity to Acrasin during the Course of Development*

The Konijn test provides a convenient method of examining the ability of cells at different stages to respond to cyclic-AMP. Vegetative and aggregative amoebae were confronted with different concentrations of cyclic-AMP, and the minimum concentration capable of showing a response is used as an index of activity. (It is impossible to test migrating amoebae because they are bound together in a pseudoplasmodium.)

Vegetative amoebae are far more insensitive chemotactically to cyclic AMP than aggregative amoebae. The minimum concentration that is weakly attractive to vegetative amoebae is 100-fold more concentrated than that for aggregative amoebae (Table 1).

There is nothing in this experiment to indicate whether or not this 100-fold increase in sensitivity appears gradually or suddenly. However, Francis (1965) has shown that *Polysphodylium pallidum* grown in liquid culture has a similar increase in sensitivity to its own acrasin and that it develops gradually over a period of many hours. Similarly, Konijn (1969) has evidence of a gradual increase in sensitivity to bacterial extract in *D. discoideum*.

TABLE 1  
CONCENTRATION OF CYCLIC-AMP SOLUTION USED IN KONIJN TEST

Sample	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup> Molar
Aggregating amoebae	—	—	—	+	+	+	—	—
Vegetative amoebae	—	+ <sup>a</sup>	+	+	—	—	—	—
Aggregateless mutants	—	+	+	+	—	—	—	—

<sup>a</sup> Plus sign indicates that there was a response.

Finally, it should be pointed out that in normal aggregation there is at least a 100-fold increase in cyclic AMP production and a 100-fold increase in the response to cyclic-AMP. This means that aggregating amoebae have an increase greater than 10,000-fold over vegetative amoebae in the stimulus-response mechanism required for aggregation.

#### *Aggregateless Mutants*

Six aggregateless mutants were tested to determine whether their inability to aggregate could be identified as either a failure to produce acrasin, a failure to produce acrasinase, or a failure to become sufficiently sensitive to acrasin, or a combination of these.

It was possible to show that the production of phosphodiesterase by the mutants was roughly comparable to that of the wild type. This was not demonstrated by the technique previously mentioned, but by a more indirect method which consists of placing a drop of cells on agar containing cyclic-AMP; as the amoebae disperse from the original confines of the drop they form a ring. As we shall see, there is evidence that this ring is caused by the presence of phosphodiesterase.

The cyclic-AMP production and the sensitivity to cyclic-AMP on the Konijn test were both low. In fact, they were exactly comparable to the condition found in the vegetative amoebae of normal, wild-type cells (Table 1). From these facts we presume that the mutants fail to aggregate because they are never able to come to that point where their cyclic-AMP production and sensitivity to cyclic-AMP surge upward; in this respect they remain permanently vegetative and therefore never aggregate. One might presume that the increased cyclic-AMP production and sensitivity are complex processes that involve numerous chemical steps and controlling genes, any one of which might be altered.

#### *Patterns of Amoeba Movement*

If a group of vegetative or preaggregation amoebae are placed in a spot on an agar surface, the cells will wander away from the original

confines of the drop. It is clear that they do not move at random, but that there is a negative chemotaxis; the cells move away from the center in an oriented fashion (Samuel, 1961). We have always assumed that a product of the amoebae directly causes this repulsion. This may indeed be the case, but there is another possible explanation which shall be briefly introduced at this point so as to clarify the basis of the experiments that follow.

The amoebae produce cyclic-AMP and phosphodiesterase, and both these substances diffuse through the agar. Since the molecular weight of cyclic-AMP is 329 and that of the phosphodiesterase is approximately 300,000 (Chang, 1968), it is possible to predict the relative rates of diffusion. The gradient will move out in proportion to the square root of the diffusion coefficient. If approximate values for their diffusion coefficients are given to these two substances based on their molecular weight, then one would expect at any one moment that the phosphodiesterase would have moved roughly one-third of the distance of cyclic-AMP. This means that surrounding a group of cells there will be an outside ring of cyclic-AMP and an inside disk of phosphodiesterase. The enzyme will destroy the cyclic-AMP in its immediate neighborhood, resulting in a gradient of increased cyclic-AMP as one moves away from the drop of cells. The exact pattern of such a gradient will depend upon many factors, such as the rate of movement of the secreting cells and the relative concentrations of the substances; yet despite these variables the ring of a gradient of cyclic-AMP increasing in the outward direction should be maintained. The hypothesis is that this could account for the oriented outward movement originally described by Samuel. There is no proof for this hypothesis, nor for the hypothesis that there is a special substance responsible for the negative chemotaxis; in fact both mechanisms could be operating. We will show, however, that if one artificially increases such acrasin gradients it is possible to get even stronger orientation of cells moving away from drops.

If cyclic-AMP is added to the agar (usually 0.05 mg/ml in the agar) and a concentrated group of cells are placed in a drop on the agar, then the amoebae will move out from the center in a strongly oriented fashion. Furthermore the amoebae, as they move out, will adhere in a ring, which sometimes separates into small clumps of cells, each of which moves out radially (see Fig. 1 in Konijn *et al.*, 1968).

The presumed explanation of the ring is that the numerous amoebae in the drop liberate large quantities of phosphodiesterase which removes the cyclic-AMP in the area below and immediately surrounding the



amoebae. This means that there will be a steep gradient of increased cyclic-AMP as one goes away from the drop radially outward into the agar. The amoebae respond to such a gradient by an oriented movement away from the center.

The fact that the cells group together in rings and droplets relates to the old observation of Shaffer (1957) that acrasin seems to induce adhesiveness of cells. The interesting point is that it would appear that it is not just the presence of cyclic-AMP which induces this excessive stickiness, but its presence in the form of a steep gradient. Cells in an even, high concentration of acrasin do not clump; they do so only in the region of a gradient.

Various methods were used to test the hypothesis that the orientation of the amoebae was the result of the activity of their phosphodiesterase on the cyclic-AMP in the agar. It is known that theophylline is an inhibitor of beef heart phosphodiesterase (Butcher and Sutherland, 1962), but it is only a weak inhibitor of slime mold phosphodiesterase (Chang, 1968). Nevertheless, if one places 0.025 mg/ml of theophylline and 0.0375 to 0.15 mg/ml of cyclic-AMP in the agar, the rings either do not form at all or are delayed many hours over the controls which lack theophylline.

Another test of the hypothesis is to use the related species, *Polysphondylium pallidum* or *P. violaceum*, species known to lack phosphodiesterase (for reasons not as yet understood). In these species there is no ring formation whatsoever.

Another fact which is consistent with the hypothesis is that the higher the concentration of cyclic-AMP in the agar, the slower the outward movement of the ring. This is to be expected, for the greater the concentration of cyclic-AMP, the more time it will take to consume it with a given amount of enzyme, and the result is a slower outward movement of the gradient.

A further test is to place a group of cells on agar containing 0.05 mg/ml of cyclic-AMP, but having regions of different thickness or depth. This is readily achieved by placing a piece of glass slide in the agar before it sets and having half the amoebae over deep agar, and half over a very thin layer which covers the glass slide. As can be seen from Fig. 3, the ring over the thin agar has moved out farther; when the agar is very thin (as it is in one area) there is no ring at all. The reason is that there is less total cyclic-AMP in the thin agar, and therefore the rate at which it is converted by the enzyme is greater, with the result that the ring moves out faster.

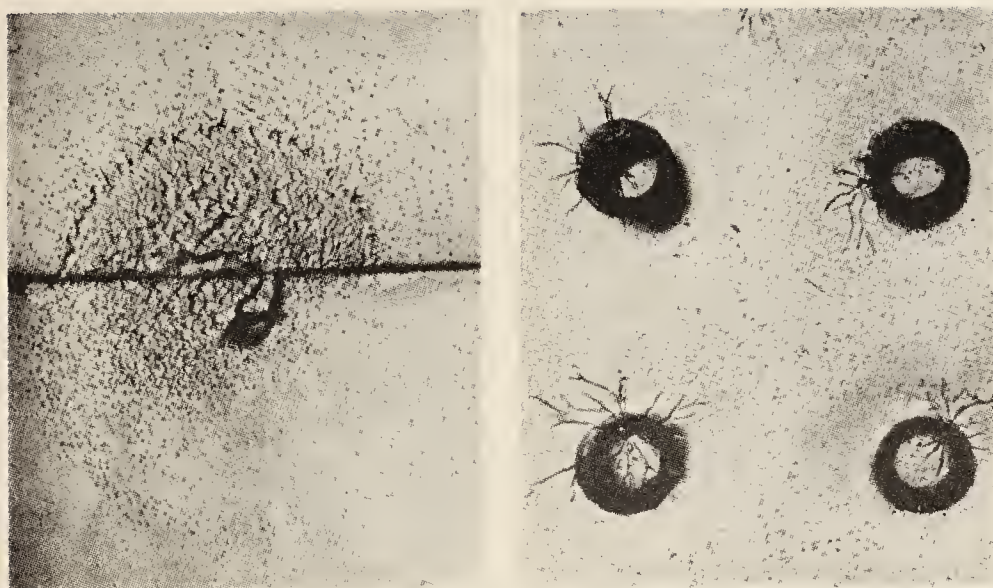


FIG. 3. *Left*: An expanding drop of cells on 0.05 *M* cyclic-AMP agar. There is a glass slide in the agar on the lower half, therefore the agar layer is very thin (especially on the right); the agar is thick in the upper half. Note that the ring expands at a greater rate over the thin agar, presumably because the lesser amount of cyclic-AMP is removed more rapidly by the phosphodiesterase given off by the cells. *Right*: A similar cyclic-AMP agar, but the agar is thin between the holes and thick over the holes. The amoebae were initially spread in an even suspension over the agar, and note that the centers form over those areas with greater reserves of cyclic-AMP.

Once this was determined, a special plastic plate was designed which was 3.5 mm thick and had many small holes drilled through it at regularly spaced intervals. Various patterns were tried, and a successful one had holes of a diameter of 1.2 mm spaced 2 mm apart. Agar containing 0.05 mg/ml of cyclic-AMP was poured over it so that it just covered the surface of the plastic. The preaggregation amoebae were deposited in an even suspension over the surface of the agar. The difficulty with the experiment was that the result depended upon having amoebae of the correct age, the correct density, and a suitable thinness of the agar between the holes. Despite these variables there were always significantly more centers forming over the holes than one would predict by chance. [32 % of the agar was over the holes and 68 % between the holes. If plain agar was used, 35 % of the centers were over the holes (17 cases) and 65 % between the holes (32 cases). With cyclic-AMP in the agar 63 % of the centers were over the holes (203 cases) and 37 % between the holes (117

cases)]. In some experiments when all the conditions were optimal, almost all the centers formed over the holes (Fig. 3). Presumably the regions over the deep holes have large resources of cyclic-AMP as compared to the surrounding thin agar region, and therefore cyclic-AMP appears to favor center formation.

In some cases, and especially evident in some experiments with another species, *Dictyostelium mucoroides*, the preaggregation cells will accumulate over the holes long before any aggregation streams appear. This, no doubt, is related to the fact that the cells always have some sensitivity to cyclic-AMP gradients; and this sensitivity increases greatly during aggregation. It is of interest to compare this to the observation of Shaffer (1962) that *D. mucoroides* is one of those species that normally grows a dense area of cells (a "cloud") before aggregation begins. The cells above the holes closely resemble these clouds.

The fact that high cyclic-AMP content seemed to stimulate center formation was confirmed in another way. A series of petri plates containing different concentrations of cyclic-AMP were evenly covered with a layer of preaggregation amoebae, the density being the same in each plate. At concentrations of 0.15 mg/ml or less there was no difference in the number of centers/cm<sup>2</sup> as compared to the controls without cyclic-AMP. But with 0.3 mg/ml there was a 6- to 8-fold increase, and with 0.6 mg/ml the number of centers/cm<sup>2</sup> increased about 20-fold. The cells failed to develop at a concentration of 1.2 mg/ml of cyclic-AMP. In other words, up to a certain limit, high concentrations of cyclic-AMP produced a large increase in the number of centers producing many small fruiting bodies, each with a minute surrounding territory.

#### DISCUSSION

We have seen that in normal development the sensitivity to, and the production of, cyclic-AMP (acrasin) increase sharply at the time of aggregation in *D. discoideum*. Furthermore, by various methods we have shown that gradients of cyclic-AMP, which orient the amoebae, are enhanced by the presence of phosphodiesterase (acrasinase), which is produced at all stages. Because the small molecule of cyclic-AMP diffuses more rapidly than the large phosphodiesterase which destroys it, a group of cells which are secreting both, will produce a gradient of cyclic-AMP which surrounds the drop of cells. This gradient can be greatly enhanced by the addition of cyclic-AMP to the agar, for the phosphodiesterase given off by the group of cells will clear the agar of cyclic-AMP only in the immediate vicinity of the cells.



This latter condition is the basis for both the acrasin test of Konijn (1965; Konijn and Raper, 1966) and the cellophane square test for acrasin (Bonner *et al.*, 1966). In the Konijn test a small drop of cells is placed on washed agar and a drop of the test substance is placed in the near vicinity. Occasionally the cells will orient toward the test drop, but the usual response is for the cells to expand radially on all sides of the drop, away from the confinement of their original circle; the washed agar holds them in unless they are stimulated by the presence of acrasin. The fact that the acrasin eventually diffuses around the drop of amoebae parallels the situation where drops of amoebae are placed on agar containing cyclic-AMP. Therefore it is presumed that it is the activity of the acrasinase in the small drops of cells that produces a gradient, and the amoebae then respond to this particular gradient by bursting out from the borders of the original drop.

The same mechanism is involved in the cellophane square test. In that case, the test substance is added directly to the agar, again surrounding the amoebae, and the rate of radial expansion of cells leaving a cellophane square placed on the agar is measured. If acrasin is present in the agar, then rings are often observed, and the presence or absence of acrasin activity is easy to detect.

If we return now to normal development, first a remark should be made concerning the production of the extracellular phosphodiesterase long before aggregation. Previously we speculated that the mechanism of aggregation was a refinement of a food-seeking device (Bonner *et al.*, 1966). The reason for such an idea was that bacteria seem to be giving off acrasin in the medium, and now we know that this is cyclic-AMP (Konijn *et al.*, 1969b). As Shaffer (1956b) pointed out some time ago, acrasin destruction by acrasinase will serve to steepen gradients and increase the potential for orientation. Therefore, we might postulate that the phosphodiesterase production in the vegetative stage serves to increase the sensitivity of the food-seeking mechanism of the vegetative amoebae. The only problem with such a hypothesis is that *Polysphondylium* seems to lack the acrasinase, yet clearly feeds successfully. The answer to this question can be resolved only when we understand the mechanism of aggregation in *Polysphondylium*.

Aggregation involves at least three aspects of the chemotactic system: acrasin, acrasinase, and the sensitivity to acrasin. At the end of the vegetative phase the amoebae are evenly distributed over the substratum; the important question is how do these three factors cause this homogeneous condition to be transformed into the heterogeneous one of

aggregation, where the cells troop into central collection points which are distributed in a nonrandom fashion over the agar. It is as though a plateau broke up into regularly spaced mountains and valleys, and we are looking for the mechanism.

Since we were unable to assess accurately the amount of acrasinase produced throughout the developmental stages, we cannot comment on its contribution to the pattern of aggregates and their distribution in space.

By contrast, both the production of, and the sensitivity to, acrasin each increase at least 100-fold during aggregation, and they begin this increase at the onset of aggregation. However, this could not contribute to the formation of the aggregation pattern if all the cells were at exactly the same level of acrasin increase or sensitivity increase. In order to break up the homogeneous pattern, the cells must differ, and at any one time some cells must be more advanced than others.

There is strong evidence that the cells at the end of the vegetative stage are not identical, but differ in their size and in various physiological ways which determine their future position in the pseudoplasmodium, as sorting out takes place in the first-formed cell mass (Bonner, 1959, 1967; Takeuchi, 1968). One would expect from this that the time of onset of increased acrasin production or increased sensitivity to acrasin would vary greatly among cells.

If this is so, then the few premature cells spotted here or there would immediately cause a discontinuity in the even distribution of cells. If one cell produced more acrasin than its neighbors, it would attract the surrounding cells. This is precisely what Shaffer (1961, 1962) has described for the founder cells of *Polysphondylium violaceum*. Furthermore, Francis (1965) showed that *P. pallidum* also had founder cells and that they increased in number with the age of his liquid culture, lending support to the idea that all the cells do not begin producing more acrasin at the same moment.

If one cell or a few cells suddenly became prematurely sensitive to acrasin, then it would be expected that they would move closer to neighboring cells. It is conceivable that, if the increase of sensitivity preceded the increase of acrasin production by some time, this might lead to the formation of clouds, so characteristic of some species for which founder cells have not been demonstrated. This is, however, highly speculative, for it is not even clear that the increased production of acrasin and the increased sensitivity can be uncoupled. All six of the aggregateless mutants showed deficiencies for both, and the intriguing question arises whether or not the two processes are linked on a biochemical level.

Another factor in the spacing pattern not yet mentioned is the ability of the first center to inhibit other centers in the immediate vicinity (Arndt, 1937; Shaffer, 1962, 1963; Bonner and Dodd, 1962; Bonner and Hoffman, 1963). There are two conflicting observations: one is that cyclic-AMP causes the disintegration of a center already formed when it is added in the close vicinity in the Konijn test (Konijn *et al.*, 1968; Konijn, 1969). The other is that, in some way not yet understood, externally applied cyclic-AMP stimulates the formation of the centers. This has been shown in the experiments where the agar containing cyclic-AMP is poured over a plastic plate containing small holes, and in this case the centers tend to form over the holes where there was a greater reserve of cyclic-AMP. The other even more obvious demonstration that cyclic-AMP stimulates center formation comes from the experiment in which it was shown that an increased concentration of cyclic-AMP in the agar produced a large number of centers per unit area (i.e., a great decrease in the territory size) so that the fruiting bodies were very small and numerous, resembling some of the "petite" mutants described by Hohl and Raper (1964).

These paradoxical results might be explained by the hypothesis that a gradient of acrasin causes the destruction or inhibition of a center (as suggested by Francis, 1965) and that an evenly distributed high concentration of acrasin results in the stimulation of center initiation.

Whatever the mechanism responsible for the formation of centers and the inhibition of secondary centers, once it has occurred the centers cause a chemotaxis which results in an uneven distribution of cells, which will produce a further unevenness in the distribution of cyclic AMP.

One began with an even layer of amoebae which were basically similar in their sensitivity to acrasin and their outpouring of acrasin and acrasinase. This uniform equilibrium condition was upset by certain cells becoming especially sensitive to acrasin and certain cells producing unusually large amounts of acrasin and simultaneously preventing other cells from doing so in the surrounding territory. Once this happened, the balance was gone, the uniformity shattered, and the new equilibrium condition is one in which all the cells are accumulated in centers or cell masses separated from one another in a spaced, nonrandom distribution.

#### SUMMARY

The purpose of this study was to examine the role in normal development of an acrasin (cyclic-AMP) and of an acrasinase (phosphodiesterase) and the sensitivity to acrasin in the cellular slime mold *Dictyostelium discoideum*.



Phosphodiesterase is secreted throughout the vegetative, aggregation, and migration stages. It is unclear whether or not cyclic-AMP is produced by vegetative amoebae, but there is a 100-fold increase between the beginning of aggregation and late aggregation, falling to a lower level during migration. The sensitivity to cyclic-AMP also increases 100-fold during aggregation, therefore the chemotactic system is at least  $10^4$  times more effective during aggregation than during the vegetative stage.

Artificial patterns of amoebae may be produced by making an uneven distribution of cyclic-AMP in the agar with an even distribution of amoebae, or the reverse. The results of these experiments show: (1) the importance of phosphodiesterase in making steep gradients (and providing the basis of various acrasin assays), (2) the role of steep gradients of cyclic-AMP in producing cell adhesiveness, and possibly inhibiting centers; (3) the center-inducing capacity of a high concentration of externally applied cyclic-AMP.

It is postulated that prior to normal aggregation, the distribution of chemicals affecting aggregation is homogeneous, and that this equilibrium is upset by sudden excessive sensitivity to cyclic-AMP and its production by certain cells, and the prevention of neighboring cells from having such an increase. The result, by chemotaxis, is an uneven distribution of cells which ultimately reach a new steady state in which they are massed together in groups spaced nonrandomly over the surface of the substratum.

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# Hormones in Social Amoebae and Mammals

*The substance that attracts social amoebae to one another to form a sluglike mass has recently been identified. It turns out that the same substance also acts as a "messenger" in mammalian cells*

by John Tyler Bonner

One of the pleasures of science is to see two distant and apparently unrelated pieces of information suddenly come together. In a flash what one knows doubles or triples in size. It is like working on two large but separate sections of a jigsaw puzzle and, almost without realizing it until the moment it happens, finding that they fit into one. I recently had this pleasure, although my own work directly concerned only one section of the puzzle. The assembling of the other section began some years ago, when a substance in the family of adenosine phosphates was found to be a "chemical messenger" intimately involved in the action of many mammalian hormones, including man's. The substance is cyclic AMP, or cyclic-3',5'-adenosine monophosphate. ("Cyclic" refers to the fact that the atoms of the phosphate group form a ring.) The relatives of cyclic AMP are, among others, the more familiar, noncyclic ADP (adenosine diphosphate) and ATP (adenosine triphosphate), substances that play key roles in plant and animal metabolism.

Cyclic AMP was discovered by Earl W. Sutherland, Jr., now at the Vanderbilt University School of Medicine, and Theodore W. Rall of the Western Reserve University School of Medicine in 1958. They and their collaborators have since learned a number of remarkable facts about the substance, the bare bones of which I shall outline here. To deal with the basic biochemistry first, cyclic AMP is formed from ATP in a one-step reaction; the enzyme responsible for the conversion is adenylyl cyclase. The substance is subject to further modification, being converted into 5' AMP (5'-adenosine monophosphate) in another one-step reaction. The enzyme that carries out this step is a phosphodiesterase specific for the reaction.

The hormone that Sutherland and Rall

first showed to be involved with cyclic AMP was adrenalin, or epinephrine, as it is now usually called in the U.S. One is taught in elementary biology that the stress of anger, pain or fear produces a great surge of epinephrine in the blood, with a resulting quick rise in the amount of blood sugar available as energy for emergency action. Inevitably one develops the simpleminded notion that the epinephrine directly splits glycogen, or animal starch, into its subunits of glucose, or blood sugar. This is far from being the case. As Sutherland and Rall discovered, cyclic AMP is implicated in a much more complex process. I shall go into a small amount of detail here because the example of epinephrine illustrates the way cyclic AMP works with hormones generally.

Skipping the step that puts the epinephrine into the blood, we can start with the liver, which is one of the places where the hormone does its work. Cells in the liver are suddenly bathed in epinephrine, brought to them by neighboring blood vessels. Adenylyl cyclase, the enzyme that converts ATP into cyclic AMP, is attached in some way to the surface membrane of liver cells. When the epinephrine reaches the cell surface, it specifically stimulates the enzyme, the conversion takes place and cyclic AMP is formed inside the cell.

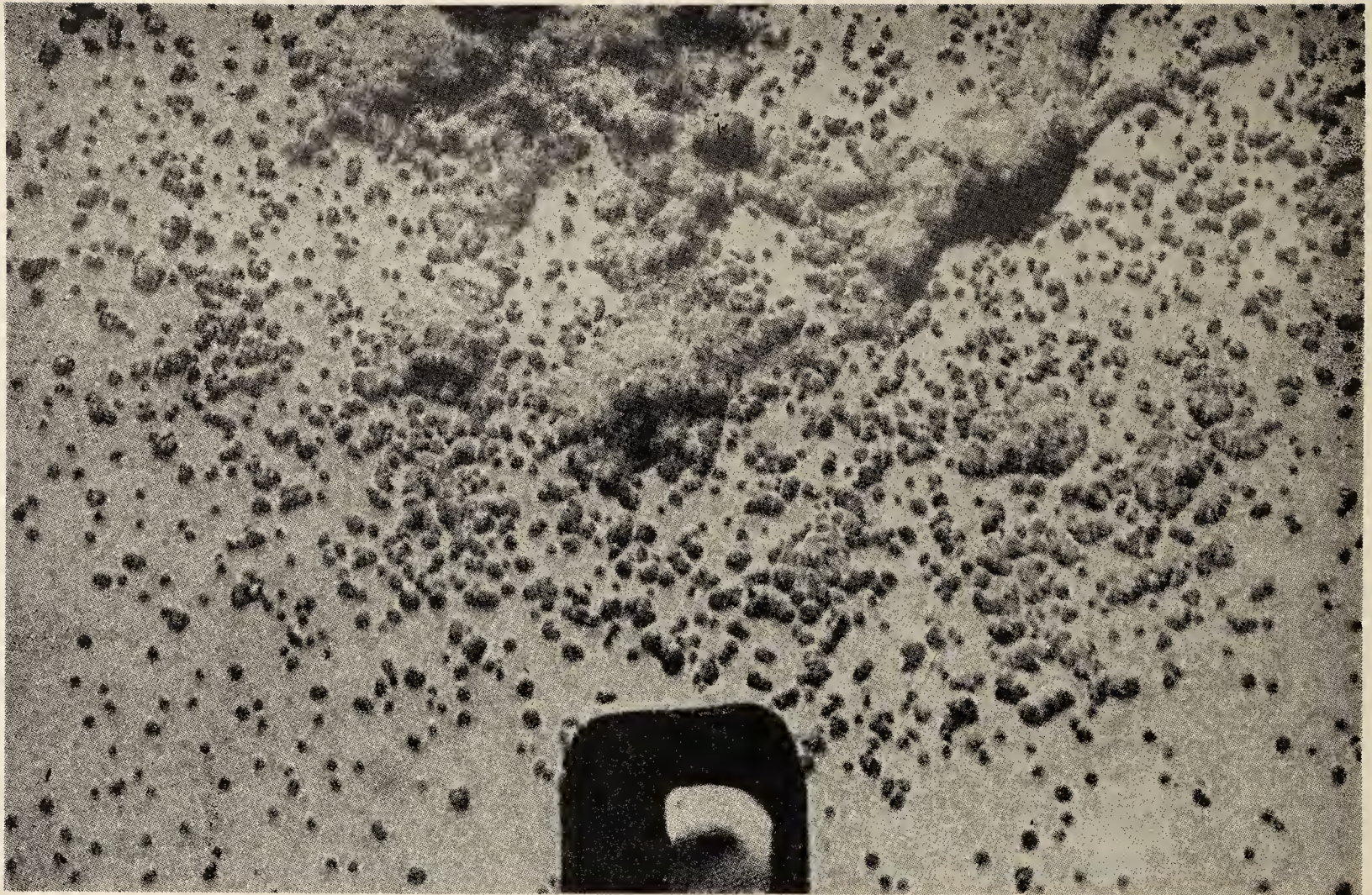
The cyclic AMP inside the cell now stimulates a second enzyme, changing it from an inactive form to an active one. Once activated, the second enzyme acts on a third to activate still another enzyme: phosphorylase. This fourth enzyme cleaves glycogen to produce glucose-1-phosphate. Before the sugar can escape from the cell, however, it must go through two more enzyme steps, one that converts it to glucose-6-phosphate and one that converts glucose-6-phos-

phate to glucose, the sugar that enters the blood [see illustration on page 81]. The entire sequence is remarkable for many reasons, not least of which is the extraordinary number of chemical steps involved in what originally was thought to be a simple process.

At the moment more than a dozen mammalian hormones are known to use cyclic AMP as this kind of "second messenger," to use the name Sutherland and his co-workers have given it. I shall not list them all here but prominent among them are glucagon and insulin, both of which are involved in blood-sugar levels, three hormones of the anterior pituitary and one of the posterior pituitary. In addition large amounts of the substance are present in brain tissue. It has been known for some time that the synaptic gaps between nerves are bridged by neurohormones such as acetylcholine. Bruce McL. Breckenridge of the Rutgers University Medical School has recently put forward the hypothesis that cyclic AMP is somehow involved in the bridging sequence.

One of the many unanswered questions about cyclic AMP is how the single enzyme adenylyl cyclase can respond to so many different hormones and do so in such a specific way. There is some evidence that the enzyme, which always appears to be associated with cell membranes, differs in different cell systems: one type can respond only to one hormone, another type only to another hormone, and so on. This raises further questions of deep interest. For example, how could such a chemical system have evolved? It would almost seem as though the fundamental part of the hormone system is the cyclic AMP, and that the variety of methods for turning on the system had evolved subsequently. I shall return to some evolutionary considerations presently, but the main basis for such a

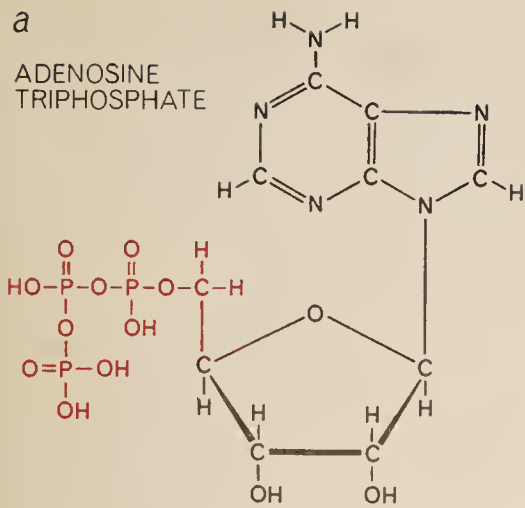




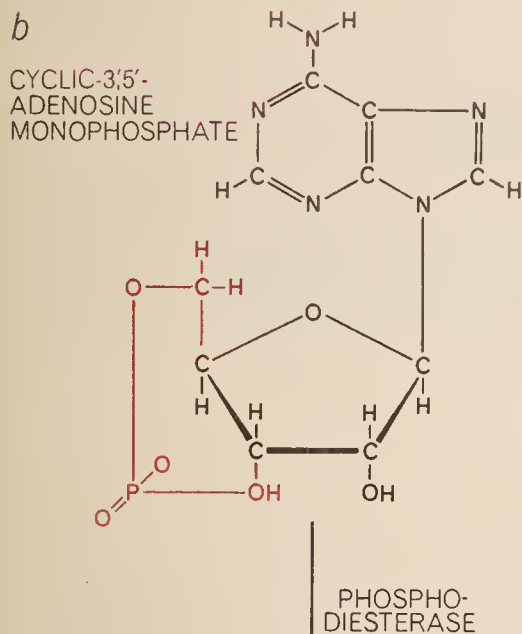
EFFECT OF ATTRACTANT on the social amoebae of the slime mold *Dictyostelium* is shown in these two photographs. In each, a small block of agar (*bottom*) contains the attractant, cyclic-3',5'-adenosine monophosphate (cyclic AMP). In the upper photograph

hundreds of amoebae have been placed near the block. In the lower photograph many have responded to the attractant by streaming toward the block. In normal development secretion of the attractant brings the amoebae together to form a multicellular organism.

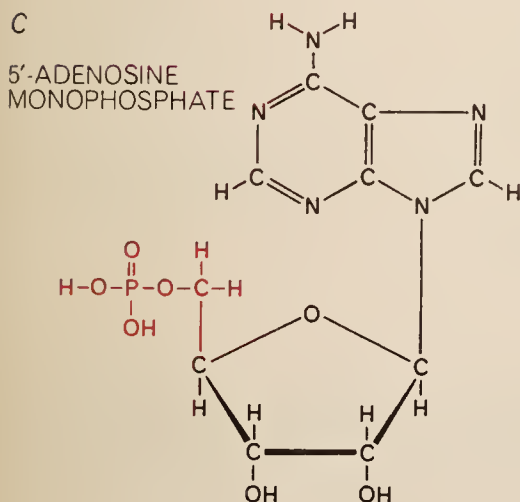




ADENYL  
CYCLASE



PHOSPHO-  
DIESTERASE



CYCLIC AMP (b) is formed from the more familiar adenosine triphosphate, or ATP (a), in a one-step reaction catalyzed by the enzyme adenylyl cyclase. The word "cyclic" refers to the ring shape of the molecule's phosphate group (color). The substance is converted into 5' AMP (c) by a phosphodiesterase that is specific for the reaction.

contention is that cyclic AMP appears to be a common component of many cells and tissues and is even found in bacteria.

I should now like to ask the reader to forget momentarily what has gone before while we look at the other piece of the puzzle. This piece involves an entire series of experiments, including some of my own, in a quite specialized area of biology. Not until the very last step, however, was there the faintest suspicion that cyclic AMP played any part in the work.

The social amoebae known as the cellular slime molds were first described by Oskar Brefeld of Germany exactly a century ago. A few years later their peculiar life history was elucidated, and today a score or more species are known. Here I am going to concentrate mainly on the species *Dictyostelium discoideum*, which was discovered in the 1930's by Kenneth B. Raper of the University of Wisconsin. *Dictyostelium* amoebae live in the soil; they play a key role in the food chain because they are the immediate predators of the soil bacteria.

In appearance and size the amoebae are similar to human leukocytes, the white blood cells, and like leukocytes they engulf bacteria by surrounding them with protoplasm. After they clear an area of food and enter a period of starvation, the individual amoebae stream together into central collection points. In a reasonably well-populated area an aggregate may number 100,000 or more cells, although if food is scarce, the number will be fewer.

The assembled amoebae gather into a cartridge-shaped mass, one or two millimeters long, which has a distinct front and hind end. The mass crawls through the cavities in the soil like a slug and shows an ability to orient toward light and heat, an ability that is completely lacking in the separate amoebae before they aggregate. As I have argued elsewhere, spore dispersal must be of key importance in the natural selection of soil organisms, and the formation of a slug and its migration to "a better place in the sun" as it wanders through the soil or among rotting leaves in the humus is therefore an important adaptation for more effective spore dispersal.

After a period of migration the slug upends itself until it points at a right angle to the surface. Some of its cells will now form a stalk, while others will go to make up a spherical mass of spores. The stalk cells swell, become hollow like the pith of a plant and die. The result is a slender, tapering hollow rod that rises a few millimeters into the air and is filled with dead cells that serve as internal

trusses. As the stalk grows the spore mass is carried aloft with it so that ultimately the delicate stalk bears a sphere of spores at its tip. Any one of the spores is capable of starting a new generation.

Because this organism is a relatively simple system of cells that differentiate (that is, take up specialized functions) it has been an object of interest to students of developmental biology for some years. One of the prime targets of study has been the social amoebae's mechanism of aggregation. How are the individuals oriented so that they stream into the central collecting points? The action is a rather pure example of morphogenesis, or formative movement, a process that is known to be most important in the initial stages of the development of all animal embryos.

At the turn of the century it was suggested that the aggregation might be due to the attraction of the individual amoebae by some kind of chemical stimulus, but there was no evidence to support this idea. Then in 1942 Ernest H. Runyon of Agnes Scott College showed that if he put an aggregation center on one side of a semipermeable membrane (such as a sausage casing) and put amoebae that were ready to aggregate on the other side, the amoebae would react. In spite of the membrane barrier they would swarm to a point on their side of the surface exactly opposite the aggregation center on the other side. Since it was known that large molecules, such as proteins, cannot readily penetrate such membranes, Runyon suggested that the chemical attractant might be a small molecule.

More evidence in support of the hypothesis of a chemical attractant was needed; it was clear that other forces, for example electric forces, could also penetrate a membrane. Studying the attractant problem further, I found in 1947 that if the amoebae began to aggregate underwater, and if one moved the water slowly past them (as though they were at the bottom of a brook), then the amoebae upstream became completely disoriented but the ones downstream oriented beautifully toward the aggregation center, even over large distances. The obvious conclusion was that aggregation was mediated by a free-diffusing agent; it might be heat or it might be a chemical substance, as Runyon had suggested. For various reasons I favored the idea of a chemical, and I gave the unidentified attractant the name acrasin because the proper name of the cellular slime molds is Acrasiales, and also because in Edmund Spenser's



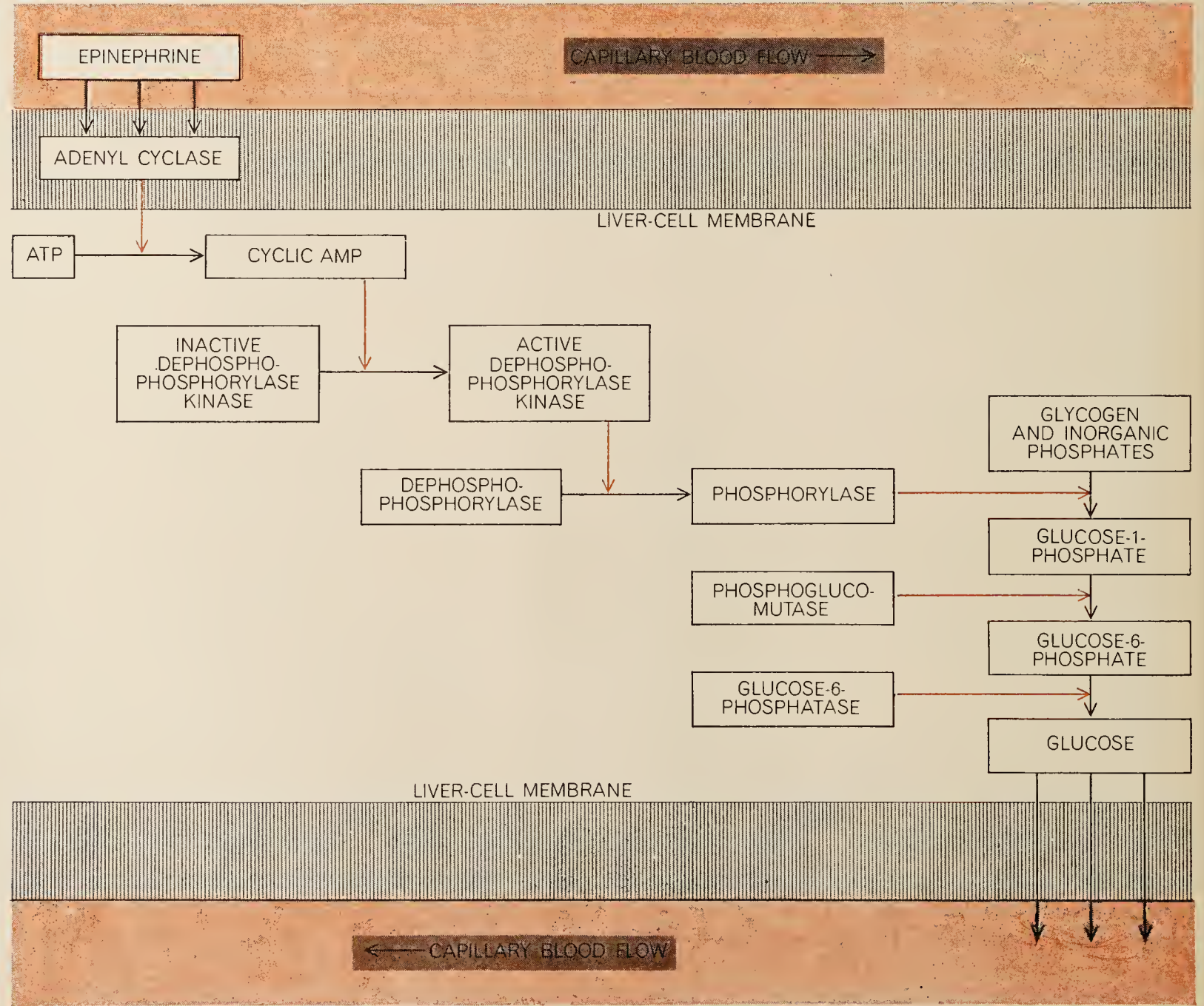
*Faerie Queene* there is a witch named Acrasia who attracted men and transformed them into beasts.

The final proof that acrasin really existed came from a remarkable set of experiments performed by Brian M. Shaffer of the University of Cambridge. At Princeton we had been unable to find any trace of the attractant at aggregation centers we had killed. Yet we felt that they must contain acrasin, because a few seconds earlier, when they were still alive, they had been actively attracting amoebae. Unknown to us, Shaffer was having the same difficulty; like us he suspected that for some reason the acrasin was rapidly disappearing.

To test this suspicion Shaffer sandwiched some active amoebae between a glass slide and a small block of agar [see top illustration on page 88]. To

the edges of the sandwich he added drops of water that had been near an aggregation center (and therefore contained acrasin) every few seconds. In a few minutes all the amoebae under the block streamed toward the wet edges. If he let a minute or more pass between drops, there was no attraction; acrasin evidently disappeared quickly and, in order to attract, it had to be applied at short successive intervals. When Shaffer froze water that had been near aggregation centers in capillary tubes and three months later used the contents of the capillaries at short intervals, the amoebae were attracted; acrasin was apparently stable at low temperatures. In any case, there was no longer any doubt about the attractant's existence. As Shaffer wrote to me after these experiments, "I have bottled it."

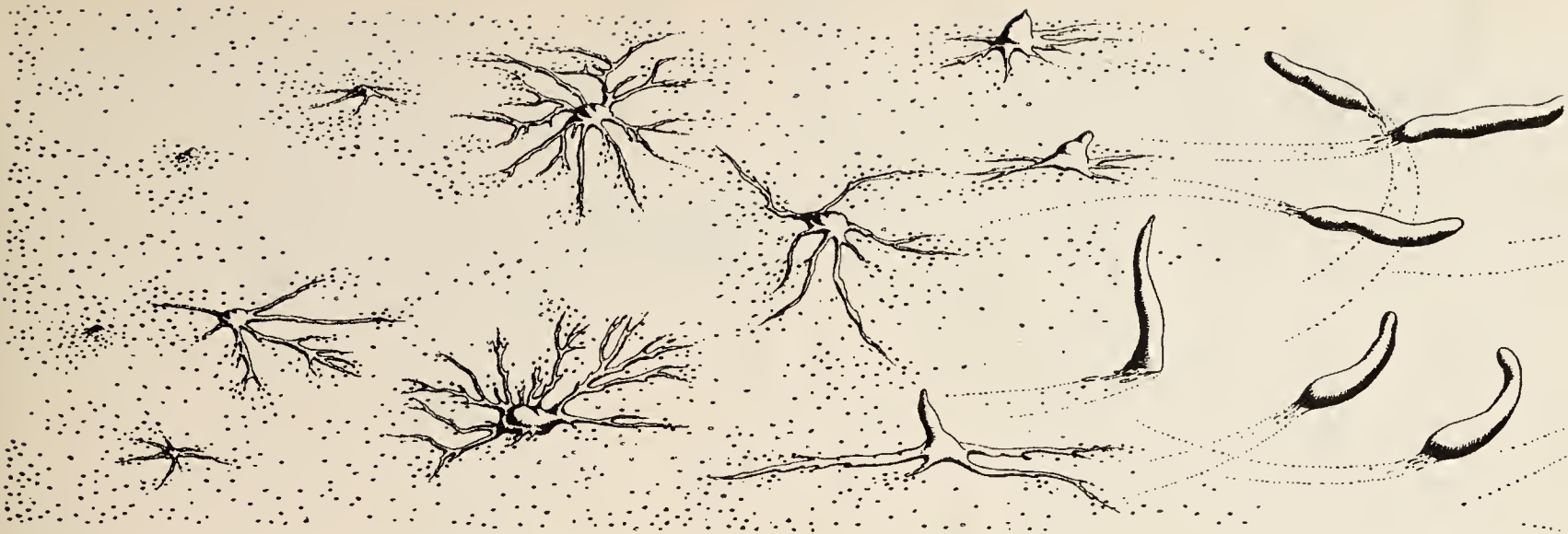
The next problem was to find out why acrasin was so unstable. Shaffer showed conclusively that the reason was that a companion substance, an enzyme, rapidly destroyed it. He did this while visiting Princeton, so that I had a close view. His principal method was to plunge water containing acrasin into methanol, which denatured the accompanying enzyme; the remaining acrasin was stable. This was an economical experiment, but I always had special admiration for what might be called his Rube Goldberg demonstration. He suspended a long cylinder of sausage casing in a moist chamber; then he painted the outside of the casing with amoebae that were about to aggregate and dripped water along the inside of the casing. The water collected in a beaker at the bottom of the casing. The acrasin it contained was stable at room



**ACTION OF SEVERAL HORMONES** in mammals utilizes cyclic AMP as a chemical messenger; illustrated here is the role of the substance in the production of blood sugar by a liver cell following stimulation by the presence of epinephrine in the bloodstream. The

enzyme adeny cyclase, attached to the cell membrane (top left), is stimulated by epinephrine and changes ATP inside the cell into cyclic AMP. The messenger substance activates a second enzyme, beginning a five-step sequence that yields glucose (bottom right).





**SOCIAL PHASE** of the life cycle of the cellular slime molds begins when individual amoebae (*far left*) begin to stream together into a

central collection point and gather into a cartridge-shaped mass that moves through the soil like a slug and is attracted to light.

temperature for extended periods of time. The membrane had passed the small molecules of acrasin but barred the large molecules of the destructive enzyme, or acrasinase, that would ordinarily have destroyed the attractant.

Shaffer's experiment with the glass slide and the agar block constituted a test or an assay for the presence or absence of acrasin. The existence of such an assay, even though it was not a quantitative one, led to a flurry of activity as workers in various laboratories attempted to determine the chemical identity of acrasin. Various substances became suspects, and I shall mention a few of the attempts that were clearly going in the right direction. Barbara E. Wright of the Retina Foundation found that urine from pregnant women contained a component that attracted the amoebae of *Dictyostelium* (it does contain such a component), but she thought this had something to do with the steroids in the urine (it does not). She and her co-workers made the interesting discovery, however, that *Dictyostelium* synthesizes rather large amounts of one steroid with an unknown function. At Brandeis University, Maurice Sussman and his collaborators found attractive substances that absorbed light at wavelengths in the ultraviolet part of the spectrum (which was on the right track), and Shaffer, then working in conjunction with organic chemists at Cambridge, went so far as to suspect that acrasin was a member of the class of compounds called purines.

The ultimate result of these various efforts was that a time came when no one was working on the acrasin problem. Simultaneously, and without knowing about each other's activities until much later, Theo M. Konijn of the Hubrecht Laboratory in the Netherlands and I

decided that since all was quiet the time had come to have a calm, noncompetitive look at the problem. We had both decided that the first necessity was to devise a truly quantitative assay, one that would not only show that an attractant was present but also measure just how attractive it was.

My test consisted in placing small cellophane squares on a dish of agar that contained the test substance; amoebae were then put on the cellophane squares and the rate at which they moved off the cellophane was recorded. At the time we thought the test had limitations (a concern that has recently proved groundless), but it taught us several things. It showed, for example, that urine from both pregnant and nonpregnant women was loaded with an active substance, and my colleague Ruth Reisberg found good evidence that the substance was a phosphate compound. It also proved that bacteria were loaded with an attractant of some kind. (This was a fact that a number of workers had

learned before. Barbara Wright had even shown the attractiveness of bacterial extracts using the Shaffer assay.) Undoubtedly the greatest benefit of my test, however, was that when Konijn read our first report, he immediately wrote to tell us of his remarkable work, which led to his coming to Princeton for a year.

Because the Konijn test was the key to later successes I shall describe it in some detail. The basic idea is to put a very small droplet of saline solution that contains amoebae on an agar plate and then to put a water droplet containing the test substance nearby. If after a few hours the amoebae spread out of the confines of their droplet, the test is scored as positive. The assay is made quantitative simply by varying the distance between the two droplets. There are, however, two key factors in making the Konijn test work. One is that the agar must be washed repeatedly and must have exactly the right degree of rigidity. Unless these requirements are met the amoebae may not stay within the confines of their droplet even though



**ABILITY OF BACTERIA** to secrete the slime mold attractant, named acrasin by the author, was discovered during the search for the substance's chemical identity; one laboratory proof is illustrated here. The collection point (*a*) of a slime mold aggregation is re-





After a period of migration the slug upends itself and some cells form a stalk that rises upward, carrying aloft a spherical mass of other cells that have turned into spores (right).

the test droplet contains no attractant.

The other key factor in the Konijn test is that the amoebae must be at just the right stage in their development in order to respond. At first Konijn would stay up until the small hours waiting for that precise moment. Then matrimony intervened, with the result that he made an interesting discovery. When he stored the test plates overnight at five degrees Celsius, within minutes after the plates had returned to room temperature the next day all the amoebae were responsive. In addition, their movements were more precisely synchronized than they had ever been before.

Working with this test, Konijn and a colleague at the University of Utrecht, J. G. C. van de Meene, began an attempt to identify acrasin. We were making a similar attempt with my test, but they carried the matter much further. We had both shown that it was unaffected by heating and that it had a negative electric charge. They were able to add the fact that the attractant had a low molecular weight (between 200 and

400). When he came to Princeton, Konijn brought along some of his purified extract. He and David S. Barkley, who was then a graduate student, determined that the attractant also absorbed light in the ultraviolet part of the spectrum, with an absorption peak at a wavelength of 259 microns.

One evening Konijn and Barkley were sitting together reviewing all they knew about acrasin—the data just noted and the fact that the substance was found in bacteria and urine. Barkley had an inspiration: Why not see if cyclic AMP was an attractant? They quickly obtained some of the substance and in no time the hunch was verified. I was in Canada at the time and received an excited telephone call from the two; they had found that cyclic AMP was amazingly effective at attracting amoebae in the Konijn test. Eventually they were to learn that as little as .01 milligram (10<sup>-11</sup> gram) was enough to make the amoebae burst out of their droplet.

This important discovery was a wonderful bit of good fortune but a big ques-

tion remained. Was cyclic AMP a naturally occurring acrasin, synthesized by the amoebae? By chance we did an incredibly stupid thing that turned out to be incredibly foresighted. At the time we happened to have another slime mold species, *Polysphondylium pallidum*, growing well in liquid culture. Without thinking, we decided to see if this species synthesized cyclic AMP. We found that it produced the substance in large quantities and that it evidently produced no other kind of attractant. This took us about a month.

We decided to complete the story by showing that cyclic AMP also attracts *Polysphondylium*. To our horror it did no such thing: the species produces the attractant but does not respond to it. This discovery raised interesting questions that are still unanswered regarding the specificity of slime mold species and also presented a puzzle: We do not know the chemical mechanism whereby *Polysphondylium* amoebae form aggregates.

It seemed as though a whole month had been wasted. To make matters worse, when we attempted to extract cyclic AMP from *Dictyostelium* we not only found that it was totally absent but also could discover no trace of any attractant of any kind. While groping in the depths of depression, one of our collaborators, Ying-Ying Chang, decided to see if our negative results were related to those that had plagued Shaffer when he first tried to "bottle" acrasin. Shaffer had shown that *Dictyostelium* produced the destructive enzyme acrasinase, and he had kept the enzyme from making the acrasin disappear by separating the two.

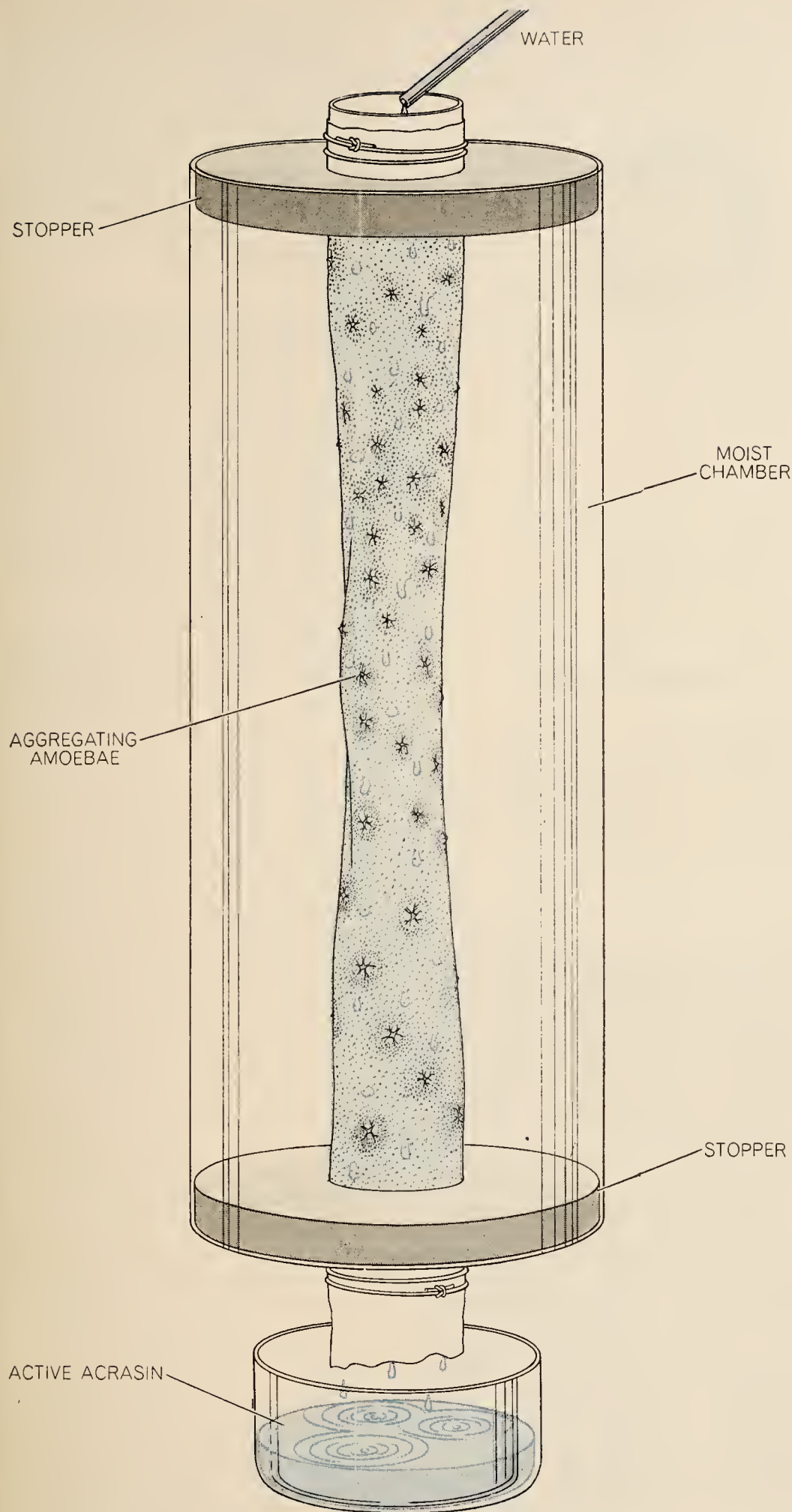
There is an enzyme (a phosphodiesterase) that changes cyclic AMP into 5' AMP. In mammals it had been isolated and partially purified by R. W. Butcher of Vanderbilt University, who was kind enough to send us some. In fact, Butch-



moved (b), leaving the stream of converging amoebae disoriented (c). A nearby clump of the common bacterium *Escherichia coli* now begins to exercise an attraction (d) and within 45 minutes the

amoebae are streaming in the opposite direction (e). The ancestors of the bacteria-devouring social amoebae probably were helped in their search for food by their prey's secretion of the attractant.





**"RUBE GOLDBERG" DEVICE** for collecting acrasin, the attractant produced by slime molds, was constructed by Brian M. Shaffer of the University of Cambridge during his visit to Princeton University. A sausage casing was suspended in a moist chamber; aggregating amoebae were placed on the outside of the casing while water was dripped down the inside. The small molecules of acrasin passed through the membrane, entered the water and were collected (*bottom*). The molecules of an enzyme that is produced by the amoebae along with acrasin and that usually destroys the attractant, however, were too large to pass through the membrane. Acrasin collected by this method remained active for long periods.

er's enzyme was one of the substances we had used to prove that the attractant secreted by *Polysphondylium* actually was cyclic AMP; when we incubated the attractant with the enzyme, all the attractant's activity disappeared. If *Dictyostelium* was producing such an enzyme, our failure to find cyclic AMP would be explained.

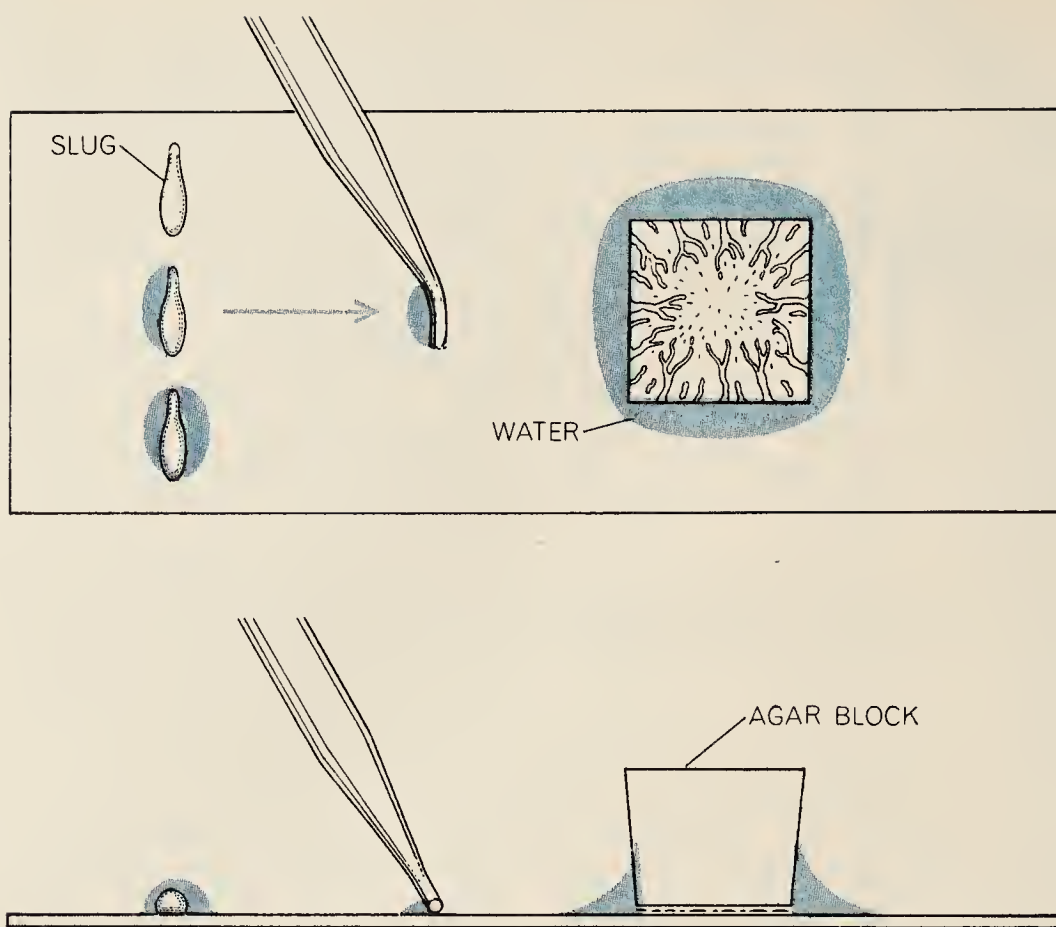
In a rather rapid series of experiments Chang showed that such was the case. *Dictyostelium* produces large amounts of a phosphodiesterase that has many (although not all) of the properties of Butcher's enzyme. The main similarity is that, like the mammalian enzyme, the slime mold enzyme breaks down cyclic AMP. The main difference is that the mammalian enzyme is found within the cell but the slime mold enzyme is almost entirely extracellular. I shall return to the significance of this fact.

Clearly Chang's work had pulled us out of our difficulties. Now all we needed was some way of proving that cyclic AMP was actively synthesized by *Dictyostelium*. We approached the problem in two ways. The first method was to grow the amoebae on dead bacteria that contained residual amounts of cyclic AMP. After a period the culture was assayed, great pains being taken to denature the destructive enzyme quickly. The assays showed that there was an increase in an attractant that had the characteristics of cyclic AMP.

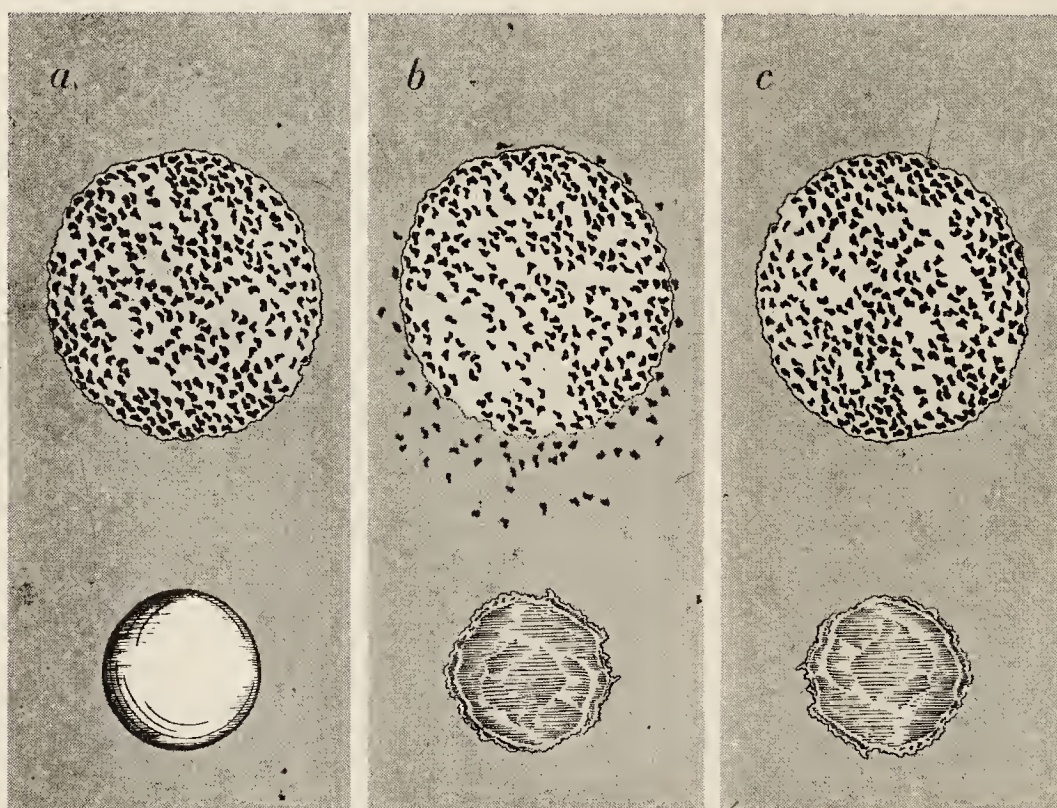
The second method, devised by Barkley, depended on separating the destructive enzyme from the attractant. One way he did this was to add beads made of a special resin to a solution containing both substances. The resin had the property of adsorbing molecules with a negative charge, such as the molecules of cyclic AMP, thus putting them out of reach of the enzyme. Another approach was a modification of Shaffer's device: amoebae were kept on one side of a semipermeable membrane and water was kept on the other. Since the membrane would not pass the enzyme's big molecules, the attractant that escaped through the membrane and entered the water was stable. The material collected in both ways was subjected to chemical analysis. Once again the only attractant of low molecular weight to be isolated was identical in characteristics with cyclic AMP. Thus we now seem to have an understanding of the basic acrasin-acrasinase system, at least in the case of *Dictyostelium discoideum*.

We have recently examined the question of when during their life cycle *Dictyostelium* amoebae produce cyclic





**QUALITATIVE ASSAY**, proving that slime mold collection centers are rich in acrasin but that the attractant is short-lived, was devised when Shaffer sandwiched amoebae between a glass slide and an agar block. He then dampened the edges of the block every few seconds with water that had been in contact with cell masses that were actively secreting acrasin; the amoebae streamed toward the dampened edges. When the applications of water were minutes apart, however, the amoebae were no longer attracted; the acrasin had vanished.



**QUANTITATIVE ASSAY**, indicating the presence or absence of acrasin and also gauging the amount present, was devised by Theo M. Konijn of the Hubrecht Laboratory in the Netherlands. A droplet containing amoebae in saline solution is put on one part of an agar plate and a droplet of the substance being tested is put nearby (a). If the amoebae emerge from their droplet in an hour or so (b), the test is scored as positive. If the amoebae stay inside the droplet (c), the test substance is deemed lacking in acrasin. The strength of the attractant may be measured by repeating the test with increased distances between droplets.

AMP. We grew the amoebae on one side of a membrane and bathed the other side with water that was collected at two-hour intervals. The water samples were concentrated and tested for the presence of cyclic AMP, using the Konijn assay. The relative strength of each sample was determined by comparing it with solutions containing known amounts of commercial cyclic AMP.

We found that when the amoebae pass through the aggregation phase there is at least a hundredfold increase in their secretion of the attractant. Simultaneously there is a hundredfold increase in the amoebae's sensitivity to cyclic AMP. This means that the whole mechanism of chemical attraction is at least 10,000 times more effective during aggregation than during the earlier stages of the amoebae's development [see illustration on page 91].

We were also able to show that, just as the amoebae continuously produce small amounts of cyclic AMP during the early stages of their development, they also continuously produce the enzyme that destroys the attractant. This mechanism of simultaneous production and destruction evidently serves an important purpose in the amoebae's life cycle. If the individual amoeba's output of attractant were not steadily eliminated, the attractant would keep accumulating until the gradient in attractant concentration that guides the individual during aggregation would be drowned out. An example is the role the destructive enzyme plays in the Konijn assay. The fact that the amoebae are initially concentrated in one spot means that a considerable amount of the enzyme is also concentrated in the same area; it destroys any attractant in the immediate vicinity of the amoebae. The result is that if attractant is applied externally, it is destroyed in the immediate region of the amoebae, producing a steep outward gradient of attractant that stimulates the amoebae to move out radially beyond the confines of their droplet.

I have pointed out that soil amoebae feed on soil bacteria and also that bacteria secrete cyclic AMP. It is quite reasonable to suppose that a positive response to cyclic AMP, among other substances, helped the solitary predators that were the ancestors of the slime mold amoebae to track their prey. Cyclic AMP is a small molecule that diffuses readily and is remarkably stable. As evolution proceeded and slime molds with multicellular fruiting bodies began to enjoy the selective advantages of more effective spore dispersal, it is possible to



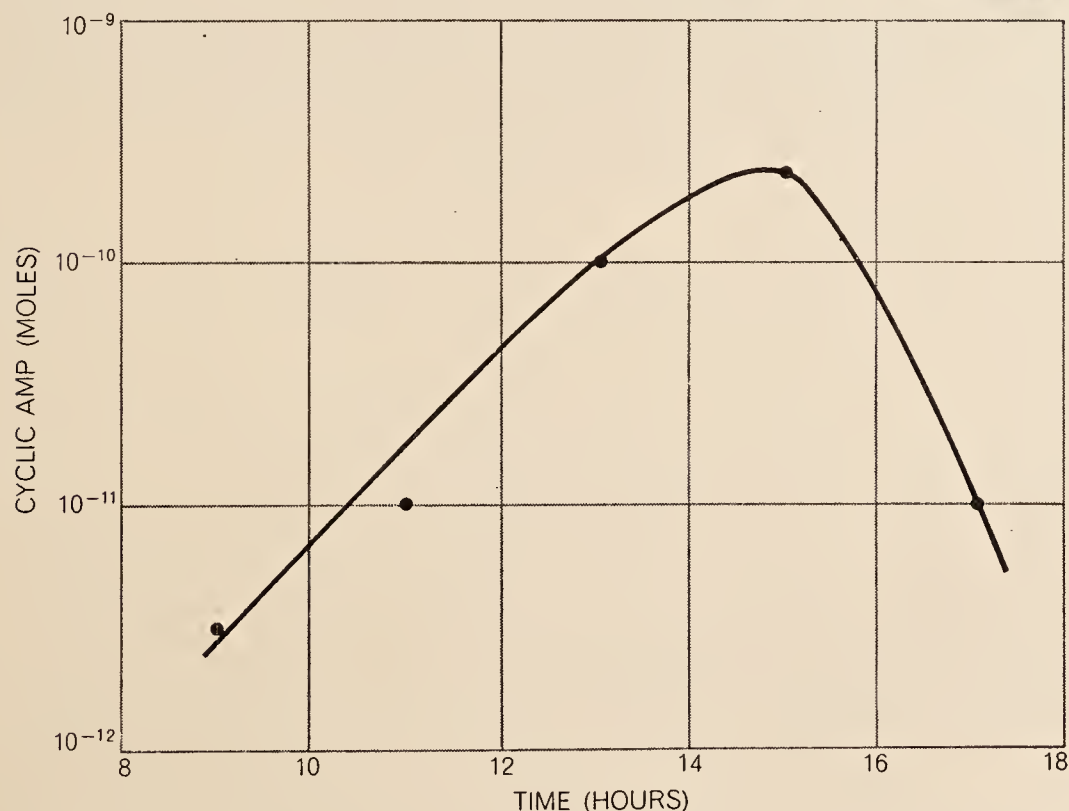
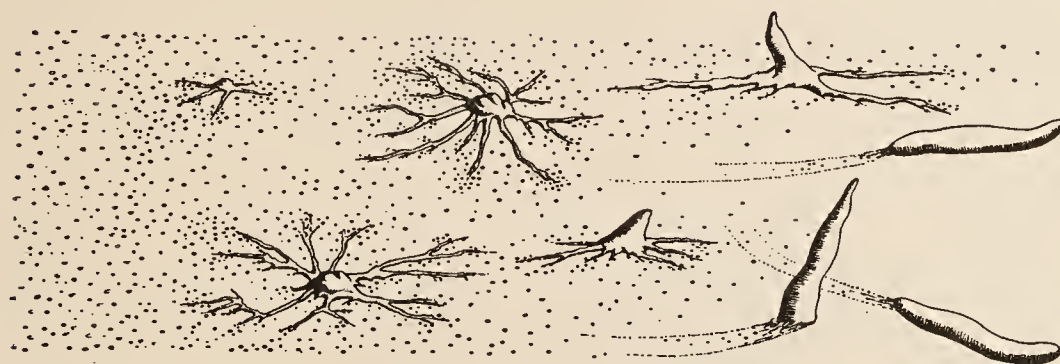
assume that social amoebae were helped to aggregate by the same mechanism of chemical attraction that already assisted their quest for food. The only required change would be to increase the sensitivity of the mechanism enormously, so that individuals would ignore their surroundings, stop hunting and swarm together instead.

Thus we find that the question of chemical attractants among social amoebae and the question of hormonal activity in mammals are connected by a single biochemical link, and that the two large pieces of the puzzle that we mentioned in the beginning do indeed fit together. There remains much to be done, however; the rest of the puzzle must be filled in.

If we look at the link, we see that in *Dictyostelium* cyclic AMP appears to be the main hormone responsible for the amoebae's social existence. We should like to know more about how it is controlled within the individual amoeba and how it orients amoebae, but its cen-

tral role is now established. Like the principal mammalian hormones, it is extracellular and provides communication between cells that are separated from one another in space. In mammals, on the other hand, cyclic AMP is triggered by the extracellular hormones and acts inside the cells. It may possibly act externally as well, but such actions have not yet been elucidated. That the chain of chemical events involving cyclic AMP in mammals is longer and more complex than it is in social amoebae is something that is to be expected of more complicated organisms.

To me, from a biochemical and an evolutionary point of view, one of the most fundamental unsolved questions is the role of cyclic AMP in bacteria. There have been some interesting beginnings on this problem, and when it is fully elucidated, I feel it will provide an important insight into the basic role of the substance in all living systems, an insight that is very much needed at this moment.



**HUNDREDFOLD INCREASE** in the amount of attractant secreted by social amoebae takes place during the seven hours in which the individual amoebae begin to stream together at collection centers. The peak is reached as aggregation is completed (*top, right of center*); production falls sharply as the fully formed multicellular slug starts to migrate.



## Induction of Stalk Cell Differentiation by Cyclic AMP in the Cellular Slime Mold *Dictyostelium discoideum*\*

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*Communicated by Arthur B. Pardee, November 28, 1969*

**Abstract.** Cyclic AMP, which is a cell attractant (acrasin) for *Dictyostelium discoideum*, will cause isolated, unaggregated cells to turn directly into stalk cells containing thick celluloselike walls and large vacuoles. From previous work we know that in the cell mass, acrasin is produced solely in the region of stalk formation during fruiting, that stalk formation involves a high level of catabolism, and that cyclic AMP stimulates catabolic enzymes in other systems. These facts obviously suggest that in the development of *D. discoideum*, cyclic AMP might be a key factor in stalk cell differentiation.

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The morphogenetic stages of the cellular slime mold begin with the aggregation of separate, independent amoebae to cell masses which ultimately differentiate into fruiting bodies with two basic cell types: stalk cells and spores. In the species *Dictyostelium discoideum*, the stalk cells are large, vacuolate, dead<sup>1</sup> cells with thick cellulose<sup>2</sup> walls, and are found both within the stalk proper and in the basal disk which surrounds the base of the stalk (Fig. 1).

Previously we have shown that cyclic AMP is a chemotactic agent (or acrasin) normally produced by *D. discoideum* and responsible for the aggregation of the cells.<sup>3</sup> Already, in 1949, I had demonstrated that acrasin was present in cell masses at later stages of development and asked the question of whether or not its presence was related to the process of differentiation.<sup>4</sup> Now, 20 years later, we can definitely answer the question: cyclic AMP does induce the differentiation of amoebae into stalk cells.

**Materials and Methods.** A haploid strain of *Dictyostelium discoideum* NC-4 (kindly supplied by Dr. K. B. Raper) was grown on *Escherichia coli* on buffered nutrient agar containing 1% peptone and 1% dextrose at 21°C for 38 hr. If the cells were still vegetative at this time, they were washed from the plate and centrifuged at  $50 \times g$  for two 5-min periods in a 1% physiological salt solution.<sup>5</sup> The tests were run in small plastic Petri dishes (50 × 12 mm—Falcon no. 1006) containing the cyclic AMP and 1% salt solution in 2 ml of 2% Difco agar. The amoebae were placed on the surface of the agar, either in drops or dispersed evenly over the entire surface.

**Results.** If the agar in the small Petri dishes contains a high concentration of cyclic AMP ( $10^{-3}$  M), and if the amoebae are placed in different size drops on the surface (3–8 mm in diameter), the cells will begin to move outward<sup>6</sup> and a strong ring will appear somewhere near the middle of this advancing edge.<sup>7</sup>

Twenty-four hours after inoculation, some of the cells in and near the advancing ring turn into separate, and often isolated, stalk cells. By 48 hours, this is

true of a large number of cells (Fig. 1). In controls with no cyclic AMP in the agar, no such isolated stalk cells are formed, but, instead, the vast majority of the cells aggregate and form normal fruiting bodies. The few remaining cells that fail to aggregate never differentiate into stalk cells. The timing of the formation of stalk cells is about the same in the experimentals and the controls.

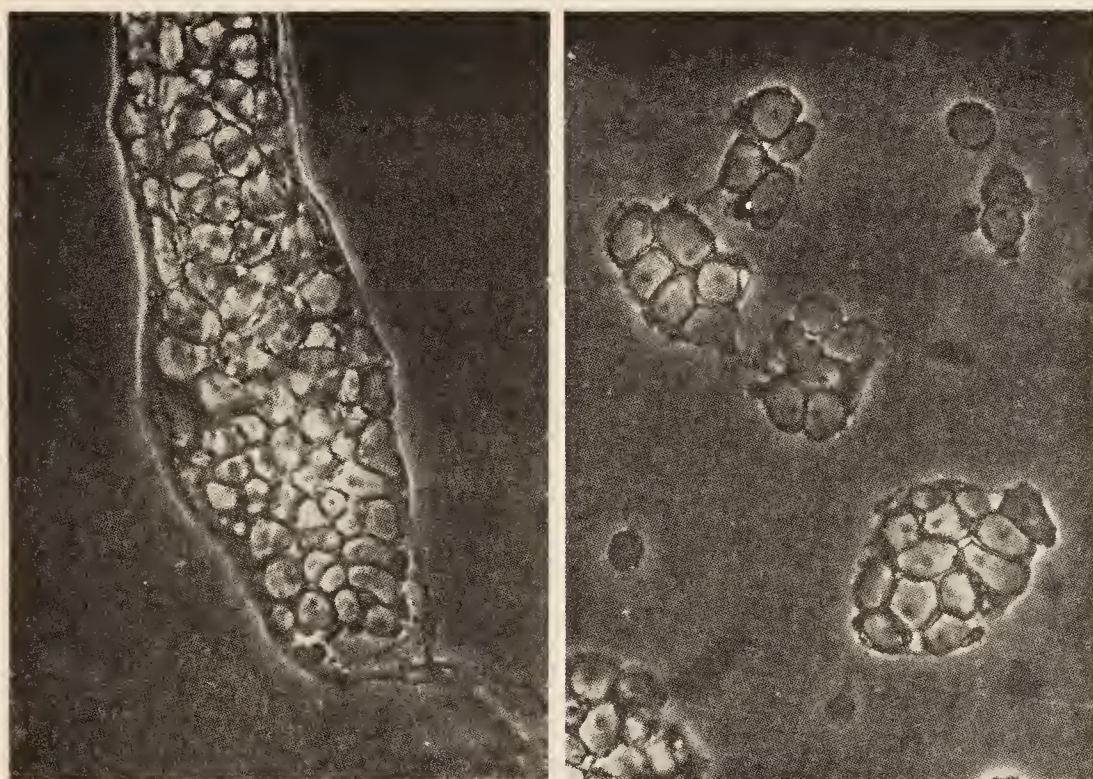


FIG. 1.—*Left*: A phase-contrast microphotograph of the base of a normal stalk on 2% agar. Reading upwards one first sees the slime sheath, then a flat basal disk, and at the top, one can see the stalk itself partially surrounded by the disk.

*Right*: A group of artificially induced stalk cells on 2% agar containing  $10^{-3}$  *M* cyclic AMP.

Both photographs are the same magnification and both were taken 48 hr after the amoebae were placed on the agar. Magnification: rounded single cell in the upper righthand corner is *ca.*  $10\ \mu$  in diameter.

The same result can be obtained by spreading the amoebae in a sparse, even layer over the surface of the agar containing  $10^{-3}$  *M* cyclic AMP. If the amoebae density is sufficiently low, the amoebae will not aggregate, and between 1 and 50 per cent of the isolated cells will differentiate into stalk cells, while none will appear in the controls lacking cyclic AMP.

It is also possible to induce stalk cells with cyclic AMP at higher densities of amoebae by adding inhibitors which interfere with normal development. If  $10^{-3}$  *M* puromycin,<sup>8</sup>  $10^{-2}$  *M* ethionine,<sup>9</sup> or  $10^{-5}$  *M* actinomycin D<sup>10</sup> are added to the agar, stalk cell induction by cyclic AMP is striking, while none of these inhibitors alone in the agar will produce isolated stalk cell differentiation.<sup>11</sup>

Because  $10^{-3}$  *M* puromycin in the agar prevents normal development, it is possible to induce stalk differentiation of isolated cells with a lower concentration



of cyclic AMP ( $10^{-4}$  M). Since the cells are producing not only their own cyclic AMP, but also an extracellular phosphodiesterase,<sup>12</sup> the cyclic AMP concentration in any one microregion of a Petri dish over the long period of time of the experiment would be exceedingly difficult to determine.

To test the possibility that a product of cyclic AMP might be active, a series of concentrations of 5' AMP were tried and found to have no effect in inducing stalk differentiation.

Some simple histochemical tests for celluloselike substances were performed to compare the composition of the cyclic AMP-induced cells with those of normal stalk and basal disk cells. Using Calcofluor white ST<sup>13</sup> (kindly supplied by American Cyanamid Co.), a fluorescent brightener, both the normal stalk cells and the experimentally induced cells showed comparable fluorescence. Furthermore they both stained positively with iodine-potassium iodide and sulfuric acid (65%) by swelling and turning distinctly blue.<sup>14</sup> Finally, the walls of the cells exhibited birefringence, a property characteristic of normal stalk and basal disk cells.

**Discussion.** It is clear from these experiments that externally applied cyclic AMP induces stalk cell differentiation in cells that have not aggregated. These cells are indistinguishable from normal stalk (or basal disk) cells; they have large vacuoles and thick celluloselike walls.

The cell mass normally produces acrasin after aggregation, and in early fruiting it is only produced at the anterior of the cell mass, in the region where the stalk cells are in the process of formation.<sup>3</sup>

Some years ago, Gregg *et al.*<sup>15</sup> showed that there was a 50 per cent reduction in certain protein fractions between the migration stage and the final fruiting body in *D. discoideum*. Furthermore they showed that some 82 per cent reduction occurred in the anterior stalk cells. In other words, these organisms are burning their own reserves for their morphogenetic phases and the largest share of such catabolism occurs primarily in the prestalk region. If we couple this information with the fact that cyclic AMP is known to promote catabolism in other systems,<sup>16</sup> we can propose that cyclic AMP induces stalk cell formation by a general stimulation of catabolism.

These different lines of evidence strongly suggest that in the normal development of *Dictyostelium* cyclic AMP induces the differentiation of stalk cells.

I would like to thank Mrs. Ellen M. Hall for her highly skilled technical assistance. I am also grateful to Drs. A. Newton, A. B. Pardee, and W. Sachsenmaier, for their helpful criticisms of the manuscript. Finally, I wish to thank Dr. F. Meins for his help with the fluorescent microscopy.

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Konijn, J. W. Mason, G. O'Keefe, III, and P. B. Wolfe, *Develop. Biol.*, **20**, 72 (1969); Mason, J. W., and H. Rasmussen, personal communication.

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<sup>5</sup> *Ibid.*, **106**, 1 (1947).

<sup>6</sup> Bonner, J. T., A. P. Kelso, and R. G. Gillmor, *Biol. Bull.*, **130**, 28 (1966).

<sup>7</sup> Konijn, T. M., D. S. Barkley, Y. Y. Chang, and J. T. Bonner, *Amer. Nat.*, **102**, 225 (1968).

<sup>8</sup> Hirschberg, E., C. Ceccarini, M. Osnos, and R. Carchman, these PROCEEDINGS, **61**, 316 (1968).

<sup>9</sup> Kostellow, A. B., Ph.D. thesis, Columbia University (1956); Filosa, M., *Anat. Rec.*, **138**, 348 (1960); Hohl, H. R., and S. T. Hamamoto, *Pacific Sci.*, **21**, 534 (1967).

<sup>10</sup> Review: Sussman, M., and R. Sussman, *Sympos. Soc. Genl. Microbiol.*, **19**, 403, see also Hirschberg, E., C. Ceccarini, M. Osnos, and R. Carchman, these PROCEEDINGS, **61**, 316 (1968).

<sup>11</sup> Some aggregation occurs in the presence of ethionine and the cell masses show some stalk cell differentiation. This is consistent with the fact that these masses are undoubtedly secreting their own cyclic AMP. Mitchell, J. L. A. (senior thesis, Oberlin College (1966)) showed that ethionine, in lower concentrations, had the effect of producing fruiting bodies with disproportionately large stalks, a matter which deserves further investigation.

<sup>12</sup> Chang, Y. Y., *Science*, **160**, 57 (1968).

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<sup>14</sup> See Raper, K. B., and D. J. Fennell, *Bull. Torrey Bot. Club*, **79**, 25 (1952).

<sup>15</sup> Gregg, J. H., A. L. Hackney, and J. O. Krivanck, *Biol. Bull.*, **107**, 226 (1954), see also Wright, B. E., and M. L. Anderson, *Biochim. Biophys. Acta*, **43**, 62 (1960); White, G. J., and M. Sussman, *Biochim. Biophys. Acta*, **53**, 285 (1961).

<sup>16</sup> For example, the recent work on cyclic AMP overcoming catabolite repression in *Escherichia coli*; see Perleman, R., and I. Pastan, *Biochim. Biophys. Res. Commun.*, **30**, 656 (1968); *J. Biol. Chem.*, **243**, 5420 (1968); Ullman, A., and J. Monod, *FEBS Letters*, **2**, 57 (1968); Monard, D., J. Janeček, and H. V. Rickenberg, *Biochem. Biophys. Res. Commun.*, **35**, 584 (1969). Also there is a large literature for mammalian systems.

## Further evidence for the sorting out of cells in the differentiation of the cellular slime mold *Dictyostelium discoideum*

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### SUMMARY

The observation of Takeuchi's that the denser cells of *Dictyostelium discoideum* tend to sort out towards the anterior end of the migrating slug (and the lighter cells towards the posterior end) has been confirmed using spore size as a method of identifying cells populations. A fraction of the anterior and posterior ends of a slug are isolated and allowed to fruit; their spores are then measured. The same is done for preaggregation cells which have been separated into heavy and light fractions, using Takeuchi's technique of centrifugation of the cells in a dextrin solution equal to the mean specific gravity of the cells. Invariably, in three experiments with different strains of *D. discoideum*, the spores derived from dense cells corresponded perfectly with spores derived from the anterior cells of the slug, and a similar correspondence was found between spores derived from light cells and posterior slug cells. Contrary to a previous view (Bonner, 1959), cell size did not always correlate with position; in one strain the anterior cells were larger, in the other two they were smaller.

### INTRODUCTION

Some years ago Bonner (1959) put forward the hypothesis that when the amoebae of the cellular slime mold *Dictyostelium discoideum* aggregated the cells were not identical but already possessed tendencies toward the formation of either stalk cells or spores, and those cells with stalk tendencies sorted out to the anterior end of the slug, while those with spore tendencies moved to the posterior end of the slug. The evidence involved the non-random distribution of mutant marker cells within the slug, and the fact that the anterior cells of the particular strain of *Dictyostelium discoideum* investigated were consistently larger than the posterior cells. Furthermore, it was shown that this size difference was probably a property the cells possessed before aggregation. This 'sorting out' hypothesis had the virtue of explaining some curious previous results such as the forward movement within a slug of grafted anterior, prestalk cells (Bonner, 1952), and the passing of the cells of a slug of one strain through the cells of a slug of another in certain grafts between species and strains (Bonner & Adams, 1958).

However, it was not until the work of Takeuchi (1969) that the sorting out

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hypothesis was given crucial and convincing evidence. First he showed that when preaggregation amoebae were stained with spore antibodies conjugated with a fluorescent dye, that some of the cells contained large quantities of spore proteins, while other cells contained little or none. Furthermore, by the time cells had formed slugs, all the cells containing the spore proteins were in the posterior, prespore region.

It is known from the pioneer experiments of Raper (1940) that by cutting a slug transversely one can induce regulation and cause prestalk cells to become spore cells and vice versa. Gregg (1965) obtained further insight into these reversible changes in spore proteins which occurred during such regulation, also using the fluorescent antibody technique.

More recently Takeuchi (1969 – this is a review of work published previously in Japanese) has marked the cells using tritiated thymidine and has confirmed the phenomenon of sorting out in detail. Furthermore, with the same cell labelling technique he has separated vegetative or preaggregation cells on the basis of their density, and he has shown that the heavier cells sort out primarily to the anterior end while the lighter cells move to the posterior end. In other words prestalk cells are heavier than prespore cells. Finally, Takeuchi (1969) has dissociated cells from different parts of the slug, and has shown, using the  $^3\text{H}$  marker technique, that the cells resume their original position when they re-aggregate and form new slugs.

It should be pointed out that Takeuchi (1963) also showed that the sorting out did not occur during aggregation, as had been previously supposed, but immediately after aggregation. At first the distribution of the two presumptive cell types is random, but later clearly segregated.

Despite the fact that the evidence for sorting out is becoming increasingly compelling, there are still a number of workers in the field who cling to the old view that the position of a cell is determined by the time it enters an aggregate. The importance of sorting out in animal embryos is now established; there is little basis, in the light of the accumulated facts, for precluding the sorting out hypothesis in any consideration of the development of the cellular slime molds.

In the work to be reported here we have additional evidence that the cells in *D. discoideum* slugs do sort out and that cell density is a significant correlate with the ultimate position of the cell within the slug, as Takeuchi (1969) contends.

#### MATERIALS AND METHODS

*Organisms.* The following strains of *Dictyostelium discoideum* Raper were used in this study: (1) Dd-1, representing the original isolate of this species, NC-4, was given to one of us (J. T. B.) in 1940 by K. B. Raper. It has been shown by Bonner & Frascella (1952) to be haploid (7 chromosomes). (2) NC-4(S): this is a small-spored strain that originated from a single spore isolated in Raper's laboratory by Dr A. T. Weber. (3) NC-4(L): this represents a large-spored strain



of NC-4, similarly isolated by Weber. Comparative studies of the two monospore cultures indicate that they are haploid and diploid respectively (Weber, 1967).

*Separation of heavy and light amoebae.* We have used the technique of Takeuchi (1969), but since he gives little specific information in his review paper, it might be helpful if we list some of the details of preparation.

Bacteriological dextrin (Type 1, Sigma Chemical Co.) was mixed with distilled water and heated to 100 °C to make a concentrated solution. Upon cooling, this was thinned by adding more water until the specific gravity (measured with a hydrometer) was 1.070. This solution was centrifuged for 40 min at 16000 *g* which cleared the solution of particles. It was then autoclaved for 30 min and stored at 5 °C.

As Takeuchi (1969) points out, different culture conditions give different mean specific gravities. His value of 1.061 was too high for our amoebae, and by further dilution with distilled water we found a specific gravity of 1.045 to be satisfactory.

The amoebae were grown at 21 °C on nutrient agar (buffered 1 % peptone and 1 % dextrose – Bonner, 1947) with *E. coli* B/r (and with some extra water added to the surface of the Petri dish). Just prior to any signs of aggregation the cells were harvested in a 1 % salt solution, and centrifuged three times to wash the amoebae free of the remaining bacteria.

The amoebae were then thoroughly mixed in 12 ml of the dextrin solution in a 15 ml centrifuge tube, and a few drops of 1 % salt solution were gently added to the upper surface so that the floating cells would not dry up during centrifugation. They were spun for 20 min at 150 *g*; in some cases this did not seem sufficient and they were spun again at 200 *g* for 20 min. Both bands were pipetted off and washed three times in 1 % salt solution, to free them of the dextrin.

*Cutting anterior and posterior fractions.* In slugs that had migrated less than a centimeter the anterior and posterior portions (*ca.*  $\frac{1}{4}$  of the whole slug) were isolated with a hair loop and allowed to fruit. Some parallel experiments were run to see if a smaller segment taken from the middle rather than the distal end of the prespore region might give different results. It was found that spore sizes of fruiting bodies derived from the posterior end and the middle of the prespore region had identical frequency distributions of size.

*Statistical significance of results.* In a previous study (Bonner, 1959) extensive significance tests comparing two populations of spores were done. It was shown that length was as effective a measure as length and width, and in all cases the means of the spore lengths derived from anterior fractions were significantly different from those of posterior fragments. To make one key check in this study, the case in which the means were closest was subjected to a *t* test. The mean lengths were 8.2 and 7.7  $\mu$  (Fig. 2), where  $t = 3.6$ , giving a  $P < 0.001$ . Clearly the difference between these means is significant, and therefore this must be true of all those cases where the difference between the means is larger.

## RESULTS

The spore length is a particularly useful way to measure cell size; not only is the length-width ratio constant on the average for spores of different size, but also one assumes that the degree of hydration of spore protoplasm is constant and therefore spore length accurately reflects the amount of protoplasm in each cell (Bonner, 1959). Others have also used spore size as a convenient marker to

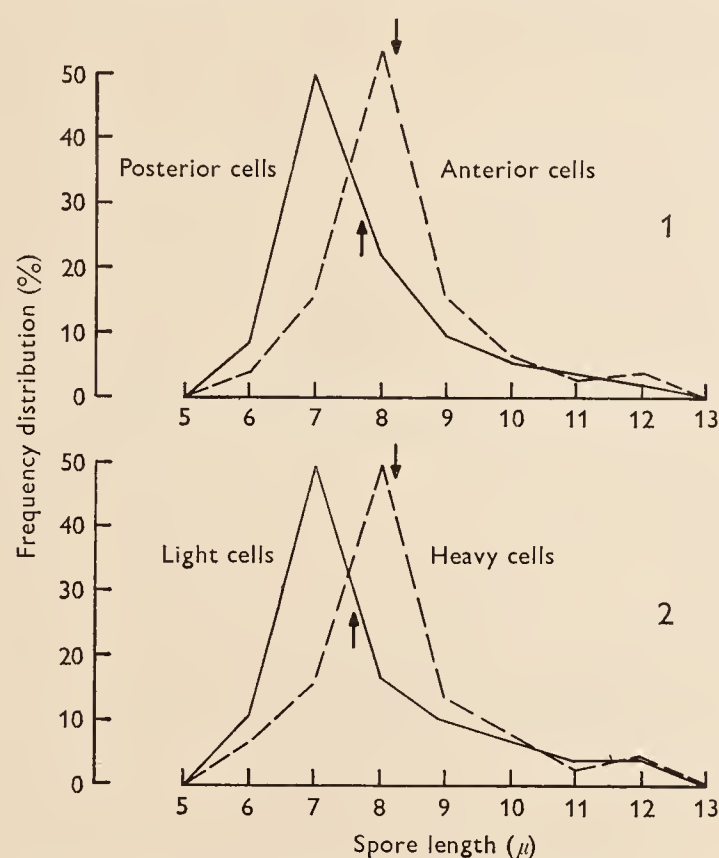


Fig. 1. *D. discoideum* – 1. A comparison of the spore lengths of 156 spores from fruiting bodies derived from posterior fractions of slugs and 165 spores derived from anterior slug fractions. The arrows indicate the mean spore lengths here and in Fig. 2.

Fig. 2. *D. discoideum* – 1. Preaggregation cells have been separated into light and heavy fractions by centrifugation in dextrin and the cells allowed to fruit. 155 spores derived from the light amoebae are compared with 178 spores from the heavy cells.

compare different cell populations (Sussman & Sussman, 1962; Sackin & Ashworth, 1969). It should be noted that in NC-4(L) the spores had the same proportions as NC-4(S) and, except in very rare instances (*ca.* 1 in 50), the same shape, unlike the diploid strain described by Sussman & Sussman (1962) which contained many sickle-shaped cells.

The technique employed here was to compare the frequency distribution of the lengths of spores derived from amputated anterior and posterior fractions of slugs with those of spores derived from heavy and light preaggregation cells

which had been separated by dextrin centrifugation following the method of Takeuchi (1969). This was done for different strains, and combinations of strains of *D. discoideum*.

#### *Dictyostelium discoideum* Dd-1

This is the same small-spored strain used previously (Bonner, 1959) and the results of the earlier experiments were confirmed; the spores derived from anterior cells were significantly larger than those derived from the cells at the posterior ends of the slugs (Fig. 1).

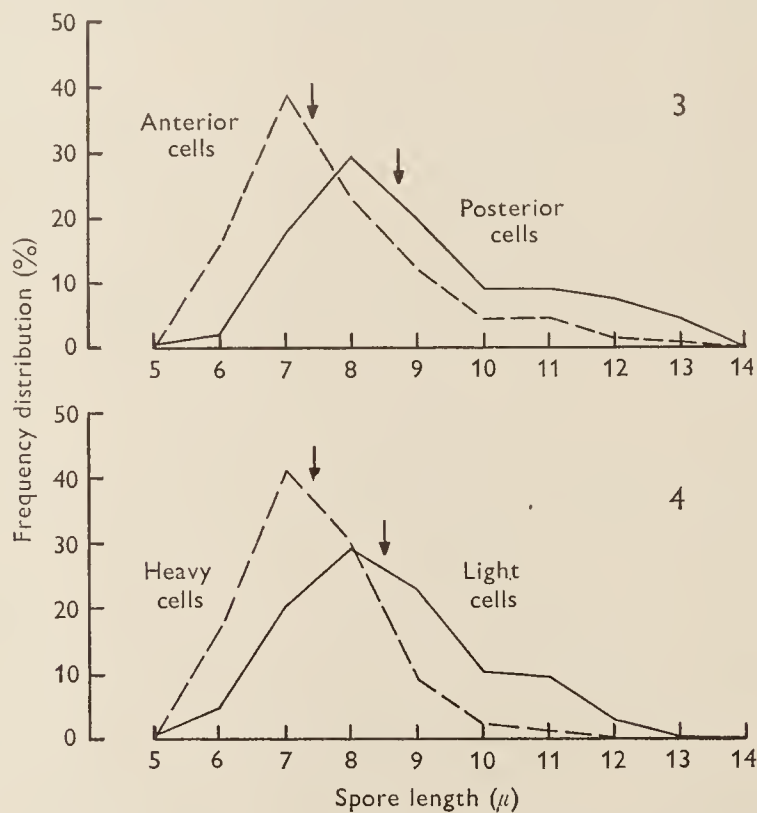


Fig. 3. *D. discoideum* – NC-4(S). A comparison of the spore lengths of 282 spores from fruiting bodies derived from anterior fractions of slugs and 289 spores derived from posterior slug fractions. Arrows indicate mean spore lengths here and in Fig. 4.

Fig. 4. *D. discoideum* NC-4(S). Preaggregation cells have been separated into light and heavy fractions by centrifugation in dextrin and the cells allowed to fruit. 337 spores derived from the heavy amoebae are compared with 369 spores from light cells.

If these were compared with spores derived from heavy and light preaggregation cells separated by centrifugation with dextrin (Fig. 2), it was clear that the heavy cells gave a spore length frequency distribution which was almost identical to the anterior cells, and the light cells were similar to the posterior cells. In other words the anterior cells of a slug of Dd-1 are larger (confirming our 1959 results) and denser (confirming Takeuchi's 1969 results) than the posterior cells.



*Dictyostelium discoideum* NC-4(S)

This strain has approximately the same size spores as Dd-1. However, when we measured the frequency distribution of spore lengths derived from amoebae in the anterior and posterior ends of the slugs, the results were the opposite from those of Dd-1. In Dd-1 the anterior cells were larger than the posterior cells; in NC-4(S) they were smaller (Fig. 3).

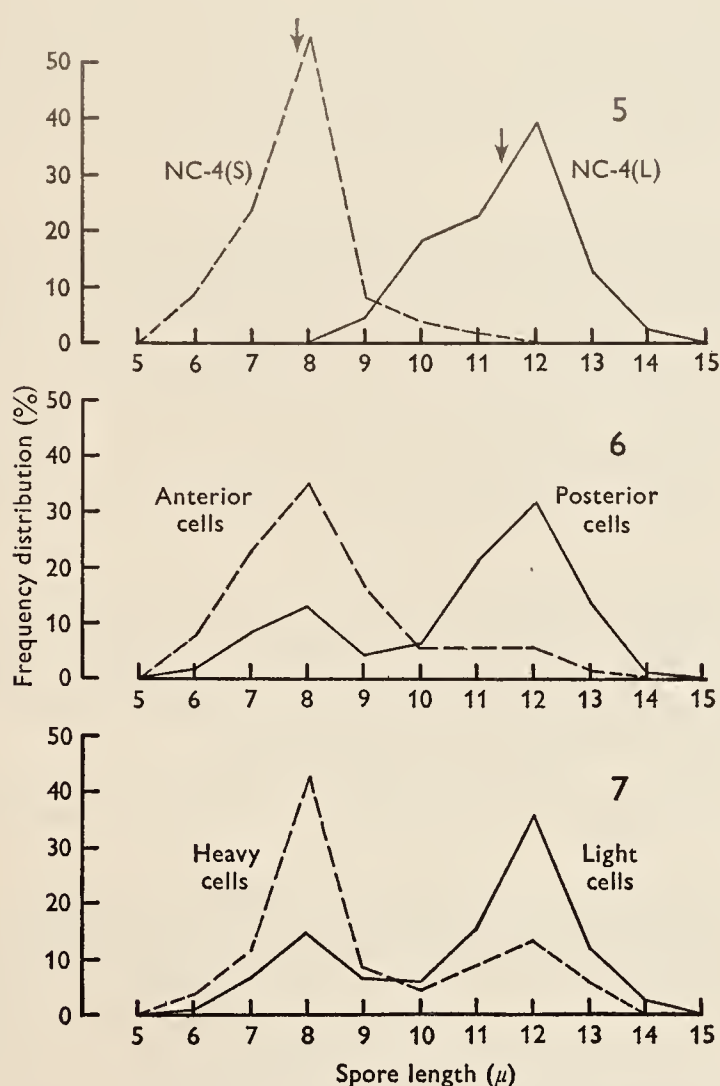


Fig. 5. A comparison of 158 spores from *D. discoideum* NC-4(S) with 158 of NC-4(L). Arrows indicate mean spore lengths.

Fig. 6. Preaggregation cells of NC-4(S) and NC-4(L) were mixed (50/50) and allowed to form slugs, and anterior posterior fractions were cut from the slugs and allowed to fruit. 446 spores derived from the anterior fractions of the slugs are compared with 390 spores from posterior slug fractions.

Fig. 7. Preaggregation cells of NC-4(S) and NC-4(L) were mixed (50/50) and centrifuged in dextrin to separate the light and heavy cells. 270 spores derived from the heavy fraction are compared with 334 spores from the light cells.

When the NC-4(S) cells were centrifuged in dextrin, there was again a result different from that obtained with Dd-1: the heavy cells produced smaller spores than the light cells (Fig. 4). Therefore in NC-4(S) the anterior cells of a slug are smaller and denser than the posterior cells. Note that when one compares the two strains, it is always the heavy cells that lie anteriorly in the slug; in one case they are the largest cells (Dd-1), while in the other they are the smallest (NC-4(S)).

*Mixtures of NC-4(S) and a large spored clone, NC-4(L)*

To test these results in another way vegetative cells of NC-4(S) were mixed approximately 50/50 with another strain derived from NC-4 which contains spores that are very much larger than NC-4(S). First we compared the two strains separately (Fig. 5); their mean spore lengths are 7.8 and 11.4  $\mu$ , which means that the volume of the larger spores is roughly three times that of the smaller ones.

If slugs were allowed to form from the 50/50 mixtures of cells of the two strains, it was possible, as before, to derive spores from anterior and posterior fractions of the slug. When their frequency distributions were compared, one can see that cells from the large spore strain predominate in the posterior end of the slug, and the reverse is true for the anterior end (Fig. 6). By comparing the two regions where the spore lengths do not overlap for the two strains, one can compute that 81 % of the spores in the anterior end were of NC-4(S) size, while in the posterior end 83 % of the spores were of the size of the larger variant (NC-4(L)).

Again the 50/50 mixture of cells at the end of the vegetative stage was centrifuged in dextrin, and the heavy cells gave rise to fruiting bodies with predominantly small cells, and the light fraction gave the reverse (Fig. 7). In this case 73 % of the NC-4(S) cells were found in the light cell fraction, and 72 % of the larger NC-4(L) cells in the heavy cell fraction (Fig. 7).

*Some preliminary tests for differences in properties between heavy and light cells of NC-4(S)*

We tested the heavy and light fractions to see: (1) if they had different chemotactic properties, using the cellophane square test (Bonner, Kelso & Gillmor, 1966), (2) how they compared with respect to the time of onset of aggregation after centrifugation, and (3) whether or not they differed in their susceptibility to cyclic AMP in the induction of stalk cells (Bonner, 1970). No differences between the two fractions were shown in any of these tests.

#### DISCUSSION

The experiments reported above offer further evidence that sorting out of amoebae does take place in the cell mass of *D. discoideum*. Furthermore, they give an independent confirmation of Takeuchi's (1969) demonstration that the heaviest cells sort out to the anterior end.

It is interesting that contrary to expectation from previous results (Bonner, 1959) cell size is not correlated with cell position; in one strain the largest cells are anterior, while in another they are posterior.

Since cell density correlates consistently with the pattern of sorting out in the three strains tested, one might ask why this should be so. One answer is obviously that it might be chance; after all a sample of three is insufficient. Even if it turns out to be a consistent correlation, it might still be that cell density has no direct bearing on the mechanism of sorting out, but only an indirect one.

Because no difference can be detected in the rates of movement of anterior and posterior cells removed from a slug (Samuel, 1961), and because heavy and light cell fractions give the same values on the cellophane square test, as shown above, one must tentatively conclude that differences in the rates of individual cell movement do not account for sorting out in cellular slime mold slugs. Instead, it might be more profitable to approach the matter in terms of differential cell adhesion, which has been so successful an hypothesis in the analysis of sorting out in vertebrate embryogenesis (Steinberg, 1970). If this turns out to be a useful approach for the cellular slime molds as well, then one must ask the question of whether or not adhesive properties and cell densities could be related in some way.

Finally, it is clear that the heavy and light cell fractions show no difference, either in terms of rate of development, or in susceptibility to stalk cell induction by cyclic AMP. This may simply be a reflexion of the fact that both these tests take a long time for completion, and by that time regulation may have nullified any differences between the two populations. This serves further to emphasize the well-known fact that until the final differentiation into spore or stalk cell takes place, the differentiation fate of a cell is reversible. It is for this reason that one must consider the preaggregation cells and the cells in the slug as showing tendencies towards stalk cell or spore differentiation; the cells have not become determined in any way and remain labile until they are fully differentiated.

It was not possible to show any functional differences between the light and heavy cells, stressing the fact that regulation is always possible in either group; they do not become determined in their differentiation until they form stalk cells or spores.

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## Folic Acid as Second Chemotactic Substance in the Cellular Slime Moulds

It has been known for some time that in certain species of cellular slime moulds acrasin, the substance which attracts the amoebae to central collection points during the aggregation phase, is cyclic AMP<sup>1-4</sup>. We were also able to show that *E. coli* gave off another substance besides cyclic AMP (henceforth referred to as bacterial factor, or BF) which attracted the vegetative amoebae of *Dictyostelium discoideum*<sup>5</sup>. Here we demonstrate that this second attractant has the properties of folic acid or one of its derivatives. We also show that folic acid and related compounds not only attract the vegetative amoebae of *D. discoideum* (No. NC-4H) but also the amoebae of six other species (*Dictyostelium rosarium* No. CC-7; *D. mucoroides* No. 11; *D. purpureum* No. 2; *D. minutum* No. V-3; *Polysphondylium violaceum* No. 1; *P. pallidum* No. 2). For the latter three species cyclic AMP is not the aggregative attractant (ref. 6 and J. T. B., E. M. H., S. Noller, F. B. Oleson and A. B. Roberts, in preparation) which raises the interesting question of whether their acrasin might be related to the folates.

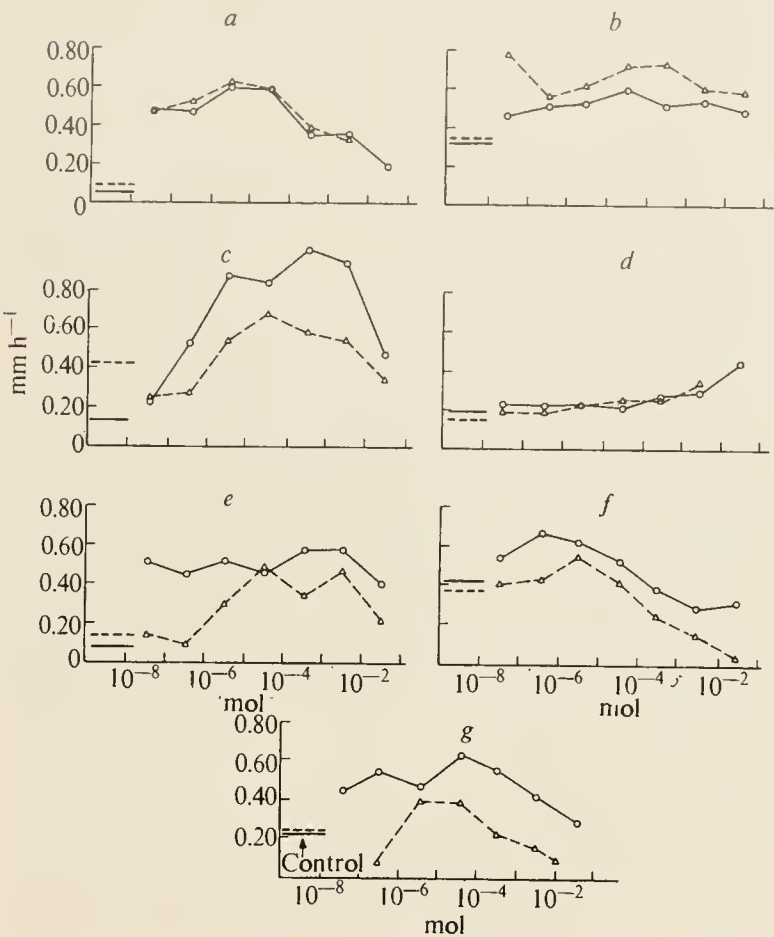
The clue to the identity of the bacterial factor came indirectly. We knew that yeast extract, milk, and urine contained an attractant which was not cyclic AMP. After removing the cyclic AMP it was possible to show, using paper chromatography (see below), that the active component from all three sources had the same  $R_F$  value as the bacterial factor in any one solvent system. Hammersten casein was similarly identified as an attractant by the Boyden test for chemotaxis with a number of species of slime mould<sup>7</sup>. Testing this and other caseins on the 'Cellophane' square test for chemotaxis<sup>8,9</sup>, using *Polysphondylium pallidum*, one can see that the activity was not in the lipid or in the most efficient extractions of the polysaccharide or protein fractions, and was absent in (Difco) vitamin-free casamino-acids (Table 1). This suggested a vitamin, and eight vitamins and cofactors commonly involved in metabolic reactions were therefore tested; of these, only folic acid was active. To confirm that folic acid was indeed the active component in casein Hammersten, the latter was chromato-

graphed on Whatman No. 1 paper; solvent was equal parts of *A* (1,245 ml. of *n*-butanol and 84 ml. of water) and *B* (620 ml. of propionic acid and 780 ml. of water) for 16 h by the descending technique in a closed tank, and the folic acid spot could be easily identified using an ultraviolet lamp<sup>9</sup>. The only portion of the chromatogram which showed any activity was the folic acid spot.

From our earlier work on the bacterial factor we knew it to be heat stable, non-dialysable, negatively charged, destroyed by acid and alkali hydrolysis, and unable to diffuse through agar. In one of the numerous chromatographic systems used for this factor we used butanol-propionic acid-water (10:5:7)<sup>10</sup> which is similar to the one mentioned above. The zone of chemotactic activity had an  $R_F$  of 0.36 and could be identified with a Hanes Isherwood perchloric acid spray. The active spot obtained from BF chromatography was further purified by ethanol precipitation. This purified BF contained large amounts

**Table 1** Chemotactic Response (using the 'Cellophane' Square Test) of the Amoebae of *Polysphondylium pallidum* (No. 2) to different Casein Preparations

Substance tested		Rate (mm h <sup>-1</sup> )
Control		0.18
Casein Hammersten	0.2%	0.38
	0.4%	0.76
Lipid extraction of casein Hammersten (chloroform-methanol, 2:1)		0.21
Polysaccharide extraction of casein Hammersten		
Ethanol	20%	0.19
	40%	0.21
	60%	0.14
	90%	0.24
Protein precipitation of casein Hammersten (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		
20%		0.26
		0.22
		0.18
		0.24
Vitamin-free casamino-acids	0.2%	0.17
	0.4%	0.19



**Fig. 1** Graphs showing the chemotactic effect of folic acid (—) and dihydrofolic acid (---) as revealed by the 'Cellophane' square test (ordinate in mm h<sup>-1</sup>) over a range of concentrations (abscissa, in mol). High values in mm h<sup>-1</sup> mean that the amoebae moved away from the 'Cellophane' square in an oriented fashion and therefore show a greater chemotactic response<sup>8</sup>. *a*, *Dictyostelium rosarium* (CC-7); *b*, *Polysphondylium violaceum* (1); *c*, *Dictyostelium mucoroides* (11); *d*, *Polysphondylium pallidum* (2); *e*, *Dictyostelium purpureum* (2); *f*, *Dictyostelium minutum* (V-31); *g*, *Dictyostelium discoideum* (NC-4H).



**Table 2** Paper Chromatography of Folic Acid and Purified Bacterial Factor

Solvent	R <sub>F</sub>	
	Folic acid	Purified bacterial factor
100% phenol water (2 : 1)	0.34	0.33
5% citric acid, NH <sub>4</sub> OH, CHCl <sub>3</sub>	0.16	0.17
5% citric acid, NH <sub>4</sub> OH, isoamyl alcohol	0.21	0.23
Na <sub>2</sub> HPO <sub>4</sub> , benzyl alcohol	0.46	0.49
5% KH <sub>2</sub> PO <sub>4</sub> , isoamyl alcohol	0.22	0.21

The first one listed above was done on Whatman No. 1 paper<sup>10</sup>; the rest on No. 2 (ref. 12). With chloroform, the ratio of aqueous phase to nonaqueous phase was 1 : 2; with other solvents the ratio was reversed. In each case 10 µg of solvent was used. The spots were identified by their fluorescence.

of polysaccharide and a small amount of folic acid. The presence of folic acid in this mixture was first demonstrated by showing maximum fluorescence at 455 nm, with excitation at 360 nm. Further, the absorption spectra of folic acid and purified BF were identical, with maxima at 280 and 340 nm. More extensive chromatographic work with the purified bacterial extract and folic acid confirmed that the unknown was folic acid (Table 2). The only portion of the chromatograms exhibiting chemotactic activity was the folic acid spot. We presume that certain of the earlier misleading information, such as the non-diffusibility of the bacterial factor in agar, is in some way the result of the polysaccharide contaminant.

The next step was to examine the chemotactic effect of folic acid. Folic acid was routinely made up with 0.1 N NaOH and dihydrofolic acid had in addition 35 mM ascorbate added to retard oxidation<sup>12</sup>. The 'Cellophane' square test plates were incubated in the dark to protect the folic acid from photolytic degradation. Tetrahydrofolic acid was not used in these studies because of its extreme instability and the fact that standard protective substances such as mercaptoethanol and dimercaptoethanol were toxic to the slime mould amoebae.

Chemotaxis was tested (1) by testing both folic acid and dihydrofolic acid<sup>12</sup> on the 'Cellophane' square chemotaxis test<sup>8</sup>, (2) by using a range of concentrations of these two substances, and (3) by doing the tests on seven different species. All the species are sensitive to both substances, although there is some variation in the range and the optimum concentration (Fig. 1). These tests were run on vegetative amoebae which were washed free of bacteria by centrifugation. All the species except *D. discoideum* began aggregating during the test, which takes a total time of about 4 h. It is possible to see attraction to folic acid in these species before and after aggregation starts.

Fig. 1, therefore, shows the response only of vegetative amoebae of *D. discoideum*, and both vegetative and aggregating amoebae of all the other species. Aggregating *D. discoideum* cells do not respond at all to folic acid, as was previously found for BF<sup>8</sup>.

Some preliminary results with folic acid precursors (biopterin and pterine) also show activity, especially noticeable with *Polysphondylium pallidum* which has the least sensitivity to folic and dihydrofolic acid (Fig. 1). This may explain why *P. pallidum* shows a greater chemotactic response to 0.4% casein (Table 1) than to these two substances.

It is known from previous work that *Polysphondylium violaceum*, *P. pallidum* and *Dictyostelium minutum* are not attracted to cyclic AMP at all, even though they produce it<sup>9</sup>. We have shown here that folic acid and folic acid precursors will attract these species. This raises the important question (which is presently being pursued) of whether or not any of the family of folic acid compounds might be the *Polysphondylium* acrasin. In any event, folic acid and cyclic AMP are being secreted by bacteria and therefore both provide a means for the feeding amoebae to find their food.

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## Immunofluorescence Evidence for the Distribution of Cyclic AMP in Cells and Cell Masses of the Cellular Slime Molds

(histochemistry/fluorescent antibody staining/cyclic nucleotide localization)

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**ABSTRACT** With immunofluorescent techniques it has been possible to show that bound cyclic AMP is uniformly distributed in the nucleus and cytoplasm of a number of species of cellular slime molds. One species (which does not respond to cyclic AMP as an acrasin) is an exception and has its cyclic AMP concentrated in the nucleus during the feeding and aggregation stage. In cell masses of *Dictyostelium discoideum* that show early signs of differentiation the anterior, prestalk cells contain more cyclic AMP than the posterior, prespore cells.

There is evidence that cyclic AMP plays at least two important roles in the development of the cellular slime molds: for some species it is the acrasin, or chemotactic agent which attracts the amoebae to central collection points to produce a multicellular organism (1), and in some species it is known to induce stalk cell formation, at least when added to isolated cells (2). For obvious reasons it would be interesting to know the distribution of bound cyclic AMP in cells and cell masses of a number of species, with special emphasis on *Dictyostelium discoideum*, which responds to cyclic AMP in both of the two ways cited above.

### MATERIALS AND METHODS

**Preparation of Cells.** The following species of cellular slime molds were used: *Dictyostelium discoideum* (no. NC-4H), *D. rosarium* (no. CC-7), *D. mucoroides* (no. 11), *D. purpureum* (no. 12), *Polysphondylium violaceum* (no. 1), and *P. pallidum* (no. 2). The amoebae were grown on a two-membered culture with *Escherichia coli* on a suitable medium (3) for 40 hr at 21°. They were then washed from the plate and centrifuged at  $25 \times g$  for three 7-min periods in a 1% physiological salt solution (4). The final suspension was brought to a concentration of  $1 \times 10^6$  cells per ml. Single drops were placed on acid-washed cover slips and spread with another. Air-dried specimens of *D. discoideum* and *P. violaceum* were prepared by placing droplets of cells ( $1 \times 10^7$  cells per ml.) on cover slips resting on the surface of 2% agar plates. When aggregation streams appeared, the cover slips were carefully washed with phosphate-buffered saline (5) and air dried before staining. To obtain migrating cell masses the washed amoebae were put directly on 2% agar plates.

**Preparation of Immunoreagent.** Antisera to cyclic AMP were obtained from randomly bred rabbits which had been previously injected with repeated doses of 2'-*o*-succinyl cyclic AMP conjugated to human serum albumin or polylysine (6). Conjugate (0.25 mg/ml) suspended in complete Freund's adjuvant was injected into each footpad. Booster injections

of 0.6 mg of conjugate were administered into the rear thighs, upper back region, and rear footpads after 6 weeks. Then 10-14 days following booster injections, the rabbits were bled from the ear. The serum immunoglobulin (IgG) fraction was obtained by ammonium sulfate fractionation (the precipitate at 40% saturation) and subsequent DEAE-cellulose column purification with 0.02 M pH 7.0 phosphate buffer. By means of radioimmunoassay using [ $I^{125}$ ]succinyl cyclic AMP tyrosine methyl ester antigen (Collaborative Research, Waltham, Mass.) (7) the various IgG fractions from the different antisera were shown to have relatively high specific titers. The  $I^{125}$  isotope was counted in an Intertechnique SL30 scintillation counter, with antibody-antigen pellet first solubilized in 0.2 ml of 0.1% sodium dodecyl sulfate. Maximal binding (35-50%) of antibody was obtained. These fractions of IgG were then used for staining the slime mold cells and sections.

**Sectioning and Staining of Cells and Cell Masses.** Migration stages of *D. discoideum* were gently lifted by their slime trail end with a fine needle and placed in tiny grooves made on the surface of 0.75-cm<sup>3</sup> 2% agar blocks. The agar was then mounted on a metal specimen holder with Ames O.C.T. Compound (Ames Co., Division of Miles Laboratories, Inc., Ind.) and the upper surface of the agar block was uniformly covered with Cryoform (Damon, IEC Division). Rapid freezing of the agar and specimens was then accomplished by packing the metal chuck into powdered dry ice. With material thus frozen, cryostat sections (5-10  $\mu$ m) of the unfixed samples were made, thawed on glass slides and briefly dried in a gentle stream of filtered air.

Sections or whole cells were then immediately stained by the indirect immunofluorescent method (8) in a moist chamber using fluorescein isothiocyanate-conjugated goat immunoglobulin (antibodies to rabbit IgG—Miles Laboratories, 64-173). Following staining, slides of cells were mounted in 90% glycerol, 10% phosphate-buffered saline and then scanned by dark field fluorescence microscopy with transmitted or incident illumination (Zeiss microscopes with BG-12 excitation filter and 470- to 500-nm barrier filters).

Various controls were run to show that the stain was specific for cyclic AMP. No staining was observed if: (1) IgG fractions from unimmunized rabbits were used as stain and (2) anti-cyclic AMP serum was incubated for 5 hr with cyclic AMP prior to use in staining. However, if other cyclic compounds, IMP, GMP, UMP, ATP, and 5'AMP (mM) were incubated with the anti-cyclic AMP for 5 hr at 4°, only slight



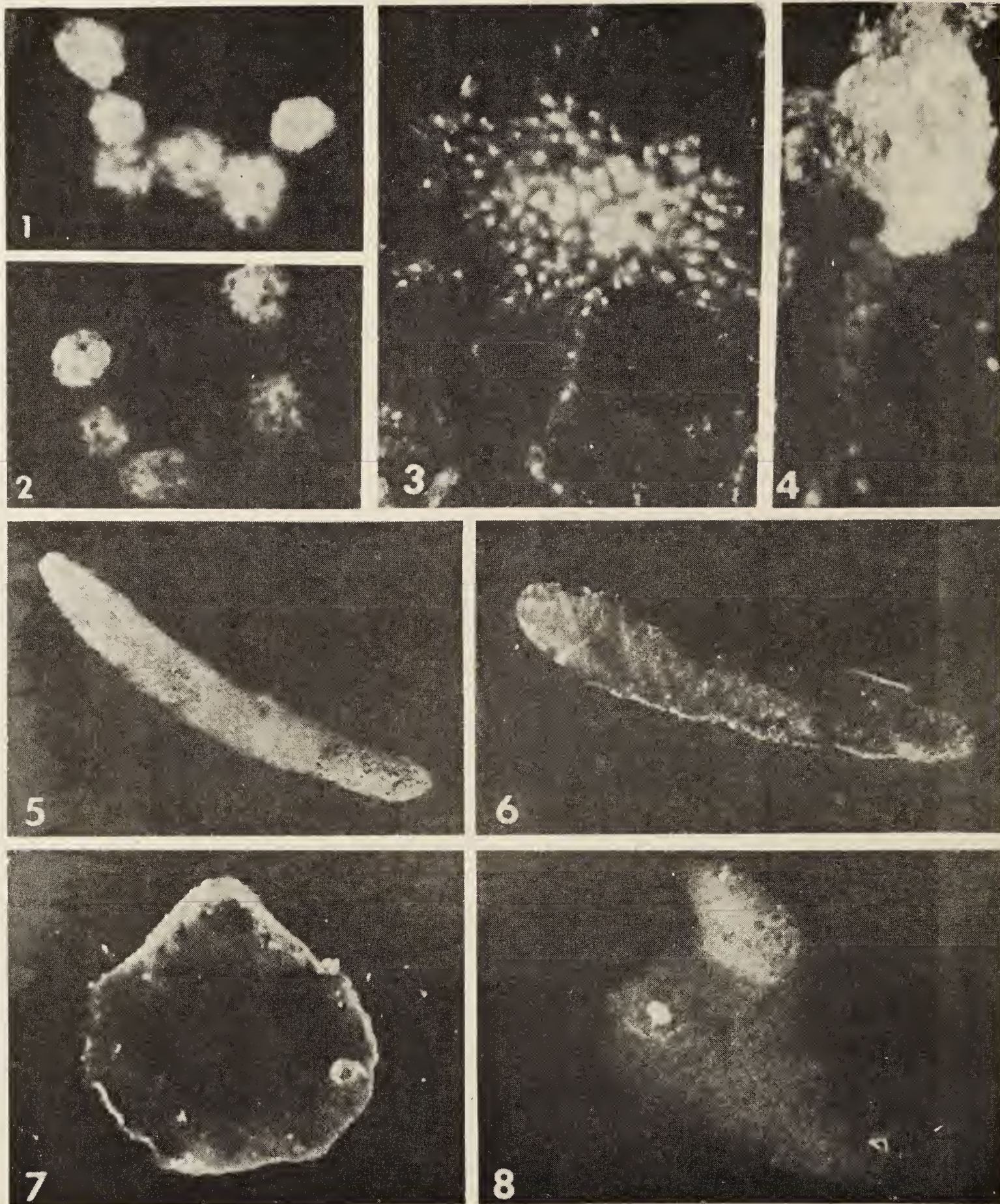


FIG. 1. The vegetative amoebae of *Dictyostelium discoideum* showing an even distribution of cyclic AMP. ( $\times 1500$ )

FIG. 2. The same for *Polysphondylium pallidum*. ( $\times 1500$ )

FIG. 3. The aggregating streams of *P. violaceum*. Note the relatively bright staining of the nucleus. ( $\times 600$ )

FIG. 4. An advanced center and a stream of *D. discoideum*. ( $\times 600$ )

FIG. 5. A longitudinal section of an early migrating cell mass of *D. discoideum*. While there is a fairly uniform distribution of the fluorescent staining, the anterior end (left) appears slightly brighter. ( $\times 150$ )

FIG. 6. A similar section of a late migrating cell mass. Note the uniform intense staining of a large mass of cells at the anterior end (left). ( $\times 150$ )

FIGS. 7 and 8. Two sections of cell masses that have righted themselves at the end of aggregation. In both cases the anterior, prestalk region shows more fluorescence than the posterior, prespore region. ( $\times 175$ )



decrease in intensity of fluorescence was observed. Also, anti-cyclic AMP IgG was preincubated with cyclic AMP and this showed no phosphodiesterase or endonuclease activity.

### RESULTS

In the vegetative cells of *D. discoideum* there is an even distribution of cyclic AMP in both the cytoplasm and the nucleus, except in the regions of the contractile vacuoles (Fig. 1). This is also true for similar preparations made of vegetative amoebae of *D. purpureum*, *D. mucoroides*, *D. rosarium*, and *Polysphondylium pallidum* (Fig. 2). On the other hand, *P. violaceum* is noteworthy in that only the nucleus shows any significant fluorescence, a condition that is particularly striking during the aggregation of *P. violaceum* (Fig. 3). In *D. discoideum* the cells remain uniformly fluorescent during aggregation, and show intense fluorescence in the central region of the aggregate (Fig. 4) due, no doubt, to the greater concentration of cells.

In the migrating stages of *D. discoideum* there is first a uniform distribution of the cyclic AMP, but later the anterior, prestalk cells show a greater concentration of fluorescence than the posterior prespore region (Figs. 5 and 6). This is especially obvious in the stages just prior to culmination (Figs. 7 and 8). In all, 34 cell masses were sectioned at these stages; of these five showed a gradient highest at the anterior end (Fig. 5), and 15 showed two distinct zones (Figs. 6, 7, and 8). It is presumed that the gradient represents an earlier condition, although there is no evidence to substantiate this.

Finally, in *P. violaceum* the cell masses show a fairly uniform distribution of cyclic AMP, and the cells are no longer merely stained in the nucleus, but in the cytoplasm as well.

### DISCUSSION

These results presumably show the distribution of bound cyclic AMP rather than the soluble form of the nucleotide. There are multiple washes during the preparation process which would have removed any soluble cyclic AMP. This is supported by the fact that the vacuoles are empty in the vegetative cells (Figs. 1 and 2).

In considering these results from the point of view of chemotaxis, those species which respond to cyclic AMP as an acrasin have cyclic AMP in their cytoplasm, as does *P. pallidum*

which does not respond to cyclic AMP. But *P. violaceum* (which is also chemotactically insensitive to cyclic AMP) shows all of its cyclic AMP in the nucleus during the aggregation stage, although by the migration stage its cytoplasm also has a uniform distribution of cyclic AMP.

From the point of view of the role of cyclic AMP in differentiation, the anterior, prestalk cells show more bound cyclic AMP than the posterior, prespore cells in *D. discoideum*. This is consistent with an earlier observation that the anterior end of the cell mass gives off more acrasin (9) and the recent finding of Maeda and Maeda (10) that before aggregation the prestalk cells of *D. discoideum* secrete more acrasin than the prespore cells. Finally, it is of interest to note that McMahon (11) predicts just such a distribution of cyclic AMP in his model of slime mold development.

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# Preliminary characterization of the acrasin of the cellular slime mold *Polysphondylium violaceum*

(chemotaxis/cell aggregation/acrasinase)

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**ABSTRACT** Some species of cellular slime mold do not respond to cyclic AMP as an acrasin, or chemoattractant. In one such species, *Polysphondylium violaceum*, we have isolated and purified its acrasin and determined some of its chemical properties, which lead us to believe it is a small molecule of less than 1500 daltons. One possibility is that it might be a peptide. The acrasin specifically attracts the amoebae of *P. violaceum* and *P. pallidum* and fails to do so for six species of *Dictyostelium* tested. We also have evidence for a specific acrasinase that inactivates the *Polysphondylium* acrasin.

Aggregation is a major event in the development of cellular slime molds: single amoebae stream together by chemotaxis and form a multicellular pseudoplasmodium. For a number of species of *Dictyostelium* (*D. discoideum*, *D. mucoroides*, *D. rosarium*, and *D. purpureum*) cyclic 3':5'-AMP is known to be the aggregation chemoattractant, or acrasin (1–3). There are, however, other species, including those of *Polysphondylium*, whose amoebae do not orient at all to cyclic AMP, and their acrasins are not known (2, 4). It is true that folic acid and other pteridine derivatives attract a variety of species (5, 6), but because they often are more effective attractants of vegetative as opposed to aggregating amoebae, and because they do not appear to be species specific, they are not obvious candidates for acrasins.

The first positive evidence that there might be a separate *Polysphondylium* acrasin came from some early experiments in which we took supernatants from aggregating *P. violaceum* amoebae that had been boiled and concentrated, and shook them with aggregation competent cells of *P. violaceum* or *D. discoideum*. The crude extract shaken with *P. violaceum* cells completely lost its ability to attract *P. violaceum* amoebae in a chemotaxis test, while the extract that was shaken with *D. discoideum* cells lost little or none of its ability to attract *Polysphondylium* cells. Since we knew from previous work that *D. discoideum* produced enzymes that inactivated the chemotactic activity of cyclic AMP (3, 4, 7–14) and folic acid and related compounds (5, 6), we concluded that *Polysphondylium* must have an acrasin of its own. This was given further support by showing that this attractant, which had been identified in this crude way, was released in peak amounts precisely during the aggregation period.

In this paper we describe the isolation of that *Polysphondylium violaceum* acrasin, its purification, and the determination of some of its molecular properties. As we shall show, one possibility is that it might be a peptide.

## MATERIALS AND METHODS

**Enzymes.** Acetylcholine esterase from electric eel, esterase from hog liver (type 1), and Pronase from *Streptomyces griseus* (type 6) with 30%  $\text{Ca}(\text{CH}_3\text{COO})_2$ , trypsin, chymotrypsin, prolidase, leucine aminopeptidase, elastase, and carboxypeptidase B were purchased from Sigma Chemical Co. Elastase was also obtained from the Worthington Company.

**Organisms.** The cellular slime mold species used were: (i) *Dictyostelium discoideum* (NC-4H); (ii) *D. mucoroides* (no. 11); (iii) *D. rosarium* (CC-7); (iv) *D. purpureum* (no. 2); (v) *Polysphondylium violaceum* (no. 1); (vi) *P. pallidum* (no. 2); (vii) *D. minutum* (V-3); and (viii) *D. lacteum* (no. 1). The amoebae of these species were grown with *Escherichia coli* B/r on nutrient agar. This was a buffered 1% dextrose and 1% peptone agar for the first five species listed above, and a 0.1% lactose and 0.1% peptone agar for the remaining three species (see p. 83 of ref. 15).

When the amoebae had just started to aggregate they were harvested and washed three times in 1% ( $10^{-4}$  M) standard salt solution (16). Species i to iv above were centrifuged for 7 min at  $75 \times g$ , and species v to viii for 7 min at  $150 \times g$ .

**Chemotaxis Test.** The cellophane square chemotaxis test was used (17, 18). Cellophane squares containing aggregation competent amoebae were placed on non-nutrient agar in small petri dishes ( $50 \times 12$  mm). In the experimentals the agar contained the sample dissolved in 1 ml of a buffer [1% salt solution (16) in 20 mM potassium phosphate, pH 6.5]; this was then added to 1 ml of 4% agar to give a total of 2 ml of agar in each petri dish. In the presence of an attractant, not only do more amoebae move off the squares than in the control, but they move further out in a given length of time. The acrasinase in the agar produces a concentration gradient of acrasin, and the amoebae become more oriented in this gradient (3) (Fig. 1).

**Isolation of Attractants from Aggregating Amoebae of *P. violaceum*.** Washed amoebae of *P. violaceum* were suspended in 50% ( $5 \times 10^{-3}$  M) standard salt solution (16) so that there were  $5 \times 10^6$  amoebae per ml. Fifteen milliliters of this suspension were added to each of a series of large petri plates ( $150 \times 15$  mm; up to 120 plates) containing 2% agar. Thirty minutes later, after the amoebae had settled down on the agar, the salt solution was carefully poured off, and the plates were air dried for 10 min and then covered. When the aggregation of the amoebae was in full swing (after 2–4 hr at  $23^\circ$ ), 10 ml of 40% ethanol in water (vol/vol) were poured on each of the plates. After 5 min the suspension was decanted and centrifuged for 10 min at  $1000 \times g$ . The supernatant was concentrated to 50 ml by boiling, and subsequently dried at  $45^\circ$ . This procedure is basically a modification of that of Shaffer (19) and Sussman *et al.* (20).

Abbreviation: PV acrasin, *Polysphondylium violaceum* acrasin.

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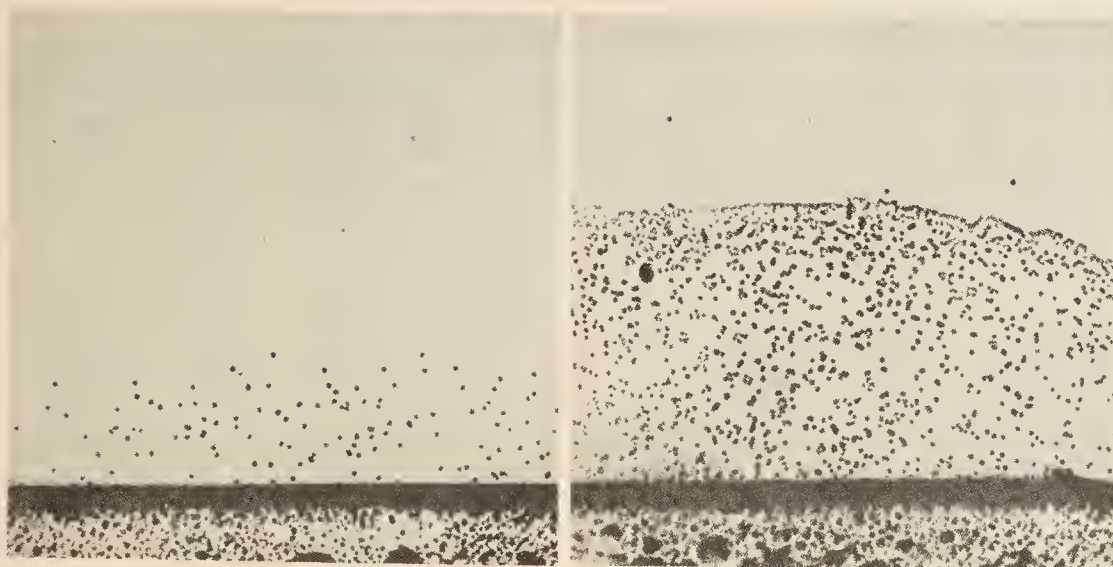


FIG. 1. The cellophane square chemotaxis test. The amoebae of *P. violaceum* moving off the edge of a cellophane square placed on 2% agar. Left: control with buffer. Right: in the presence of *P. violaceum* acrasin (1/1000 part of one entire collection). (Width of each photograph is about 2 mm.)

The chemotactic activity of the crude material and the fractions obtained in the purification procedure were determined by subjecting small aliquots (1/1000 to 1/100 part of total) to the chemotaxis test using *P. violaceum* amoebae.

## RESULTS

### Fractionation and purification of *P. violaceum* acrasin

**Anion Exchange Chromatography on QAE-Sephadex.** The dried raw fraction of attractants was extracted twice with 4 ml of H<sub>2</sub>O and centrifuged for 10 min at 10,000 × *g*, and the combined supernatants were passed over a column of QAE-Sephadex anion exchanger (Sigma Q-25-120, Cl<sup>-</sup>-form, prewashed with H<sub>2</sub>O, bed volume 3 ml). The column was then washed with 6 ml of H<sub>2</sub>O at pH 5 or at pH 8, and most (more than 90%) of the chemotactic activity did not bind to the anion exchanger. The active fraction was called Q1.

However, some additional chemotactically active material can be eluted from the QAE-Sephadex with 20 mM HCl (fraction Q2), indicating the presence of another attractant which is negatively charged. Also, in earlier experiments, when the attractants were extracted from aggregating amoebae with 70% or 100% ethanol (a procedure which breaks the cells), yet another attractant could be eluted from the QAE-Sephadex between 100 mM and 200 mM HCl (fraction Q3). Fractions Q2 and Q3 were not purified further for they were not species specific.

**Cation Exchange Chromatography on SP-Sephadex.** Fraction Q1 was subsequently applied to a column of SP-Sephadex cation exchanger (Sigma SP-C25-120, H<sup>+</sup>-form, prewashed with H<sub>2</sub>O, bed volume 3 ml). The chemotactic activity passes the column with H<sub>2</sub>O (fraction SP). In a similar experiment the cation exchanger was equilibrated with 20 mM HCl, and fraction Q1 in 20 mM HCl was applied. The chemoattractant did not bind to the cation exchanger and passed the column with 20 mM HCl.

**Gel Filtration on Sephadex G-15.** Fraction SP was concentrated to 2.4 ml at 45° and applied to a column of Sephadex G-15 equilibrated with H<sub>2</sub>O (Pharmacia, diameter 2.6 cm, height 57 cm). The compounds were eluted from the column with H<sub>2</sub>O. The flow velocity was 120 ml/hr, and fractions of 10 ml were collected. The void volume of the column was 105 ml; the attractant was eluted between 170

and 190 ml (fraction G), and inorganic ions, like [Co(H<sub>2</sub>O)<sub>6</sub>]<sup>++</sup> or [Cu(H<sub>2</sub>O)<sub>4</sub>]<sup>++</sup>, peaked at 210 ml. Fraction G was concentrated to dryness at 45°.

The elution volume of the chemotactically active material was not changed if elution was conducted with 20 mM HCl instead of H<sub>2</sub>O, showing that none of the *P. violaceum* attractant absorbed to the Sephadex matrix in H<sub>2</sub>O (21).

Because the attractant eluted far behind the exclusion volume but before the inorganic ions, its molecular weight is probably less than 1500.

**Partition Chromatography on Cellulose Thin Layers.** Dried fraction G was dissolved in 1 ml of H<sub>2</sub>O and applied to a cellulose thin-layer sheet (Eastman Chromogram Sheet 6065 Cellulose). After the chromatogram was developed with 1-butanol saturated with 0.5 M HCl, it was divided into seven horizontal fractions. Each fraction was scraped off the sheet, extracted twice with 3 ml of H<sub>2</sub>O, and dried.

The fraction with chemotactic activity moved to a *R<sub>F</sub>* between 0.70 and 0.86. We call this fraction *Polysphondylium violaceum* or PV acrasin, and it was used in most of the experiments described below. (It should be mentioned that cyclic AMP and folic acid stayed close to the origin, with *R<sub>F</sub>* values below 0.1.)

**Adsorption Chromatography on Activated Silica Gel Thin Layers.** The PV acrasin could also be chromatographed on activated silica gel thin layers (Baker-flex Silica Gel 1B) using pure ethanol as a solvent (22). Fractions were again scraped off the sheet and extracted with H<sub>2</sub>O. The chemotactic activity moved to the fraction between *R<sub>F</sub>* 0.44 and 0.56.

### Further chemical characterization

In the following experiments a solution of purified PV acrasin was divided into several samples of equal volume and dried at 45°. The chemotactic activity was tested in aliquots of the sample before and after the experiment. The reaction mixture was also rechromatographed on cellulose thin layer (see above) and then tested to see if the acrasin molecule was still active.

**Heat Stability.** Dry samples of PV acrasin were incubated for 10 min in an oven at 100, 120, 140, 160, 180, and 200°. In no case was there any loss of chemotactic activity.

**Solubility.** Dry samples of PV acrasin were extracted twice with 3 ml of one of the following solvents: water,



methanol, ethanol, pyridine, acetone, 1-butanol, dioxane, ethylacetate, chloroform, and ether. The extracts were centrifuged; the supernatants were dried at 45°, subsequently dissolved in buffer, and applied to the chemotaxis test.

The PV acrasin is soluble in water, methanol, and ethanol; slightly soluble in pyridine, acetone, 1-butanol, and dioxane; and insoluble in ethylacetate, chloroform, and ether. Clearly, the attractant is polar.

**Ionic Properties.** PV acrasin does not bind to an anion exchanger (equilibrated with 20 mM imidazole-HCl buffer, pH 8), nor to a cation exchanger, either in H<sub>2</sub>O (pH 5) or in 20 mM HCl. (These experiments were performed both with crude extracts and purified PV acrasin.) It is therefore likely that PV acrasin is not an anion, a cation, or a zwitterion (the latter because of the result with 20 mM HCl).

**Determination of Free Amino Groups.** When a Dowex Ag 50WX8 (H<sup>+</sup>-form, 100-200 mesh) column was used, the active PV acrasin passed through in 0.1 M phosphate buffer (pH 7.0), indicating the absence of a free amino group. As a control, a similar column held 0.01 M of tyrosine when eluted with the buffer, but was released with 0.1 M ammonium hydroxide.

**Gel Electrophoresis.** This lack of any significant overall charge on the molecule was confirmed by gel electrophoresis, first in 0.015 M barbital-0.015 M Tris (pH 7.5) and then in 0.03 M Tris (pH 6.0). Bromophenol blue (0.1%) was used as the tracking dye in the first instance and pyronin B (0.1%) in the second. In experiments lasting 2 hr at 4° with 3-4 mA per gel, there was essentially no movement of the PV acrasin in the polyacrylamide gels.

**Periodate Oxidation.** A dry sample of PV acrasin was dissolved in 2 ml of water, and 0.75 ml of 100 mM sodium periodate was added. The reaction mixture was kept in the dark for 2 hr and then passed over a column of AG 1-XB (200-400 mesh, Cl<sup>-</sup>-form, Bio-Rad, bed volume 3 ml) in order to remove iodate and excess periodate (23). The eluate was concentrated at 45° and then rechromatographed on a cellulose thin-layer plate.

After periodate oxidation the chemotactic activity moved to the same *R<sub>F</sub>* region as before, indicating that the acrasin molecule is neither inactivated nor altered by periodate. The presence of vicinal hydroxyl groups in the PV acrasin can therefore be excluded, which in turn rules out all common sugars as a functional part of the molecule.

**Alkali and Acid Lability.** Dry PV acrasin was dissolved in 5 ml of water and divided into five equal parts. One part was boiled at neutral pH for 30 min. By addition of 1 ml of 40 mM K<sub>3</sub>PO<sub>4</sub> the second part was brought to pH 12 and incubated for 1 hr at 25°. The third part was brought to pH 12 in the same way and heated for 20 min at 80°. The fourth part was mixed with 1 ml of 2 M HCl (yielding a final concentration of 1 M HCl) and incubated for 1 hr at 25°, and the fifth part was mixed with 1 ml of 2 M HCl and boiled for 30 min. After the incubation period each sample was adjusted to pH 6.5 for the chemotaxis test.

The PV acrasin is an alkali-labile molecule; incubation at pH 12 for 1 hr at 25° caused a decrease of the chemotactic activity, and heating at this pH for 20 min at 80° lead to complete inactivation. Incubation of the acrasin in 1 M HCl for 1 hr at 25° did not lead to a loss of chemotactic activity. However, boiling in 1 M HCl for 30 min caused complete inactivation. Boiling at neutral pH for 30 min did not affect the acrasin.

**Esterases.** Dry PV acrasin was dissolved in 4 ml of 20 mM imidazole-HCl buffer pH 8.0 and divided into four samples

of equal volume. The samples were incubated for 1 hr at 25° with one of the following preparations dissolved in 1 ml of the same buffer: (i) 0.5 mg of acetylcholine esterase; (ii) the same inactivated by boiling; (iii) 1 mg of hog liver esterase; and (iv) the same inactivated by boiling. All the samples were boiled after the incubation period and centrifuged; each sample was chromatographed on cellulose thin-layer plates as described previously. In all instances the full chemotactic activity was recovered from the appropriate *R<sub>F</sub>* region of the plates. These enzymes did not affect the acrasin (even though they inactivated acetylcholine in control experiments).

**Proteases.** A number of proteases were tested in the same manner, and similar negative results were obtained with trypsin, chymotrypsin, elastase, prolidase, leucine aminopeptidase, and carboxypeptidase B. Also, there was no activity loss of PV acrasin when incubated with insolubilized trypsin, chymotrypsin, and papain. However, clear inactivation was obtained with peptidase from hog intestinal mucosa and Pronase from *Streptomyces griseus*. The latter was investigated carefully, and it was possible to show, in four separate experiments (using 5 mg of Pronase), that the chemotactic activity was totally eliminated and not recoverable in any part of the thin-layer plates, while the normal recovery appeared in the controls with the boiled enzyme. In order to be certain that the PV acrasin was not simply binding to the enzyme, the mixture was incubated at 4° instead of 22°, producing no acrasin inactivation. Finally, the Pronase was dialyzed against 0.05 M EDTA to eliminate neutral proteinase activity (24); it then was no longer capable of inactivating the PV acrasin. Therefore, one could assume that some specific neutral proteinases in *S. griseus* Pronase are able to destroy the acrasin activity.

**Weight/Protein Analysis.** One entire collection of PV acrasin (from 100 large petri plates; see *Materials and Methods*) was purified through to the cellulose thin-layer step. Its total weight was 2.1 mg. With the Folin-Ciocalteu test for protein, a yield of 650 µg was obtained, using bovine serum albumin as a standard.

The material was hydrolyzed under reduced pressure with 1 ml of 12 M HCl for 20 hr at 110°. In some preliminary experiments the hydrolysate was examined in an amino-acid analyzer. There were considerable background amino acids, but the active material contained some proline, valine, and leucine. There was less clearcut evidence for threonine, glutamine, alanine, and isoleucine.

## Specificity

When we had isolated and purified the attractant it was important to determine if it was specific for *P. violaceum*. In the following experiments it was possible to show that indeed it was specific for both *P. violaceum* and *P. pallidum*, but did not attract the amoebae of any of the *Dictyostelium* species tested.

(i) Extracellular material was isolated and purified from aggregating amoebae of *D. discoideum* using the procedure described for *P. violaceum*. None of the material from any of the fractions attracted sensitive *P. violaceum* cells; *D. discoideum* does not give off PV acrasin.

(ii) Purified PV acrasin, when incubated with aggregation competent amoebae of *P. violaceum*, lost its activity, most likely by the action of an acrasinase (see below). However, when a similar experiment was done with *D. discoideum* amoebae there was no loss of activity; *D. discoideum* does not inactivate PV acrasin.



Table 1. Chemotactic response of aggregation competent amoebae of eight species of cellular slime molds to PV acrasin and cyclic AMP

Species	Chemoattraction by	
	PV acrasin	cyclic AMP
<i>P. violaceum</i> (no. 1)	+	0
<i>P. pallidum</i> (no. 2)	+	0
<i>D. discoideum</i> (NC-4H)	0	+
<i>D. rosarium</i> (CC-7)	0	+
<i>D. mucoroides</i> (no. 11)	0	+
<i>D. purpureum</i> (no. 2)	0	+
<i>D. minutum</i> (V-3)	0	0
<i>D. lacteum</i> (no. 1)	0	0

For each species at least two chemotaxis tests were performed. +, Strong chemotactic response. 0, None; same as buffer control.

(iii) Purified PV acrasin was tested on sensitive amoebae of a number of different species (Table 1), and it is clear that it only attracts the two *Polysphondylium* species. Chemotactic results with cyclic AMP are also included in Table 1. Note that two small species, *D. minutum* and *D. lacteum*, are attracted neither by cyclic AMP nor by the PV acrasin.

#### Acrasinase

These experiments were designed to determine whether or not *P. violaceum* produced an enzyme (or enzymes) capable of inactivating PV acrasin, and whether the enzyme was present outside or inside the amoebae, or bound to a particulate fraction.

Aggregating amoebae and surrounding extracellular material were washed off of 12 large petri dishes (2% agar; 150 × 15 mm) with 30 ml of 20 mM potassium phosphate buffer in 1% salt solution (pH 7.0) (16). To separate the various fractions, we centrifuged the cell suspension for 10 min at 1000 × *g*, yielding a supernatant and a precipitate of cells. The supernatant was subsequently centrifuged for 20 min at 30,000 × *g*, resulting in a clear supernatant (extracellular fraction). The precipitate of cells was washed twice with buffer and centrifuged for 10 min at 1000 × *g*. Precipitated cells (0.7 ml) were suspended in 5 ml of buffer, treated in a Branson sonifier 5 times for 10 sec, and subsequently centrifuged for 10 min at 1000 × *g*. The supernatant was then centrifuged for 20 min at 30,000 × *g*, yielding a new supernatant (intracellular fraction) and a precipitate of cell fragments. The precipitate was washed by suspending it in 5 ml of buffer, centrifuging for 10 min at 1000 × *g*, and subsequently centrifuging the supernatant for 20 min at 30,000 × *g*. Again the precipitate was suspended in 5 ml of buffer (particulate fraction).

The three fractions were divided into two parts, and one part of each fraction was boiled. PV acrasin samples were incubated with these fractions or with buffer for 30 min at 25°. Subsequently the reaction mixtures were boiled and centrifuged, and the supernatants tested for chemotactic activity.

All three fractions have the capacity to inactivate the acrasin. Furthermore, the inactivating principle is heat labile and loses its ability to inactivate upon boiling. Therefore, it is likely that the inactivator in all three fractions is an enzyme, an acrasinase. This situation is similar to that of *D. discoideum*, where the occurrence of extracellular (7–14), intracellular (4, 25), and membrane-bound cyclic cAMP-phosphodiesterases (12–14) have been demonstrated.

#### DISCUSSION

To summarize the results briefly, we have found a substance that appears to act as a specific acrasin for *Polysphondylium*; there is also a specific acrasinase produced by *Polysphondylium violaceum* which, along with the acrasin, is not produced by *Dictyostelium discoideum*. No known species responds chemotactically to both cyclic AMP and PV acrasin.

This *Polysphondylium violaceum* acrasin has a molecular weight of less than 1500 and is heat stable. It is polar but uncharged; it is not an anion, or a cation, or a zwitterion. The inactivation of acrasin by alkali and acid in comparatively mild conditions suggests the presence of an ester bond, an esterized carboxyl group of an amino acid, or a labile peptide bond [such as the  $\gamma$ -glutamyl bond in glutathione (26)].

One possibility is that the substance is a peptide. The best evidence comes from the Pronase results. If it is a peptide, it must have a blocked amino group. This raises the possibility of a cyclic peptide, or the group could be blocked in some other way, as Schiffmann *et al.* (27) have shown for leukocyte attractants.

Besides the further identification of the PV acrasin molecule, another approach will be important. It is well known from earlier work that *Polysphondylium* species synthesize and release cyclic AMP, as well as a cyclic AMP-phosphodiesterase (4, 10). What is the biochemical relation between the PV acrasin and the cyclic AMP? Does PV acrasin act as a primary messenger and cyclic AMP as a secondary messenger in a manner similar to what one finds in many mammalian hormone systems? This question, which is being actively pursued, is of interest both from the point of view of cell physiology and from that of phylogeny when one considers that in *Dictyostelium*, cyclic AMP seems to serve both functions.

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# Cell differentiation in *Dictyostelium* under submerged conditions

(oxygen/polarity/pattern formation)

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**ABSTRACT** Hitherto it has not been possible to obtain spore and stalk cell differentiation of the cellular slime molds in submerged cultures. It is shown here that cells, when placed in roller tubes under an atmosphere of oxygen, will form clumps and differentiate in 48–72 hr into mature spores and stalk cells. Although differentiation occurs without the normal morphogenetic movements, there is the appearance of an anteroposterior polarity of the cells in the clump. In addition to oxygen we examined a number of other factors that affect differentiation.

It has always been assumed that differentiation in the cellular slime molds could not occur under water, for development would invariably stop at aggregation (1), and that an air–water or an oil–water interface was required. The success of an oil–water interface, which was first demonstrated by Potts (2), has been ascribed to the fact that mineral oil can be saturated with 10 times more oxygen than can water. As will be shown, if oxygen is added to roller tubes containing *Dictyostelium discoideum* cells suspended in a physiological salt solution, then clumps of cells will form that show both mature spore and stalk cell differentiation. This work was first presented at a meeting in May, 1976 at Cold Spring Harbor; at the same meeting I. Takeuchi reported similar results in roller tubes using different methods. Ultimately, the findings of both these studies will increase our understanding of the mechanism of cell differentiation in the cellular slime molds.

## MATERIALS AND METHODS

All the experiments reported here were done with *Dictyostelium discoideum* NC-4 grown on *Escherichia coli* B/r on 1% peptone–dextrose buffered agar. The amoebae were washed three times by centrifugation at  $75 \times g$  in  $10^{-2}$  M salt solution (1). Two milliliters of a cell suspension ( $5 \times 10^6$  cells per ml) were put into 15-ml test tubes with ground-glass stoppers. The tubes were flushed out with 20 volumes of 100% oxygen (Liquid Carbonic Co.) before they were sealed with silicone grease. (Earlier experiments were done with screw-cap test tubes sealed with Parafilm.) The tubes were placed on a Multi-Purpose Rotator (Scientific Industries, Inc.) and rotated at 18 rpm at a low angle to the horizontal at  $21^\circ$  in the light.

Two fixation methods were used: (i) 2% glutaraldehyde followed by 1%  $\text{OsO}_4$  (both in  $10^{-2}$  M salt solution), and (ii) 2% glutaraldehyde plus 1%  $\text{OsO}_4$  in 0.1 M cacodylate buffer at pH 7.2. For light microscopy, specimens were embedded in methacrylate and thick-sectioned with a Porter-Blum microtome. The sections were stained with the periodic acid-Schiff (PAS) stain for non-starch polysaccharides (3). For electron microscopy Epon was used for embedding and the thin-sectioning was done on an LKB Ultramicrotome III. The electron microscope used was a JEOL JEM-100C.

Abbreviation: PAS, periodic acid-Schiff.

## RESULTS

### Time course description of differentiation

One of the most interesting aspects of this study has been the morphological events that accompany the differentiation of the clumps in the roller tubes. The following description, which is also shown in diagrammatic sequence in Fig. 1, is based on clumps that have been cultured in  $10^{-2}$  M salt solution under an atmosphere of 100% oxygen. The observations described below were made on living material stained with a vital dye (neutral red) and fixed preparations stained by the PAS method for nonstarch polysaccharides. These stains allow one to distinguish between the prestalk and prespore regions (4, 5).

Small clumps of less than 100 cells form in minutes after the roller tubes are started, and by 2 hr they have coalesced into larger clumps of about 100–200  $\mu\text{m}$  in diameter. At this time the individual cells show differences in their PAS staining, but the dark and light cells are randomly distributed. By approximately 8 hr, the outermost cells form a shell that is exclusively prestalk. (This would be the terminal state if the clumps were under air rather than oxygen.)

By 10–12 hr many clumps become somewhat elongated, and at the same time there is an accumulation of debris, which first appears in the center of the clump. If Sephadex beads (40  $\mu\text{m}$ ) are added to the tubes, then these are also incorporated into the center of the clumps and later extruded along with all other debris in a sticky matrix at one end of the clump. The matrix has the general appearance of slime sheath material. Both the vital dye-stained and PAS-stained material show that the prespore cells lie at the end of the clump that secretes the debris and the prestalk cells are at the opposite end. At this time, while the entire prestalk region is pure prestalk, a few prestalk cells still remain intermixed in the prespore region.

This situation persists until about 18 hr, by which time the outer shell of prestalk cells becomes quite rigid. (This can be easily demonstrated by using a fixative that causes shrinkage; there will be a clear separation between the rigid prestalk shell and the interior.) Also by this time the prespore region is made up of exclusively prespore cells. It is clear that an anterior-posterior polarity is established just as in the normal migrating slug. There is an anterior prestalk region and a posterior prespore region with attached slime sheath material. The only difference from normal slugs is that there is a cortical layer of prestalk cells that surrounds the entire clump.

By 22 hr final differentiation begins. The outside cells in the cortical shell start to differentiate into mature stalk cells, and this progresses inwardly, thickening the stalk cell shell. By about 36 hr mature spores start to appear in groups in and around the innermost stalk cells of the thickened cortex. Both spores and stalk cells continue to mature, but even by 72 hr the larger clumps still have a small slug-like structure with prespore and prestalk zones near the center of the fully differentiated mass of cells.



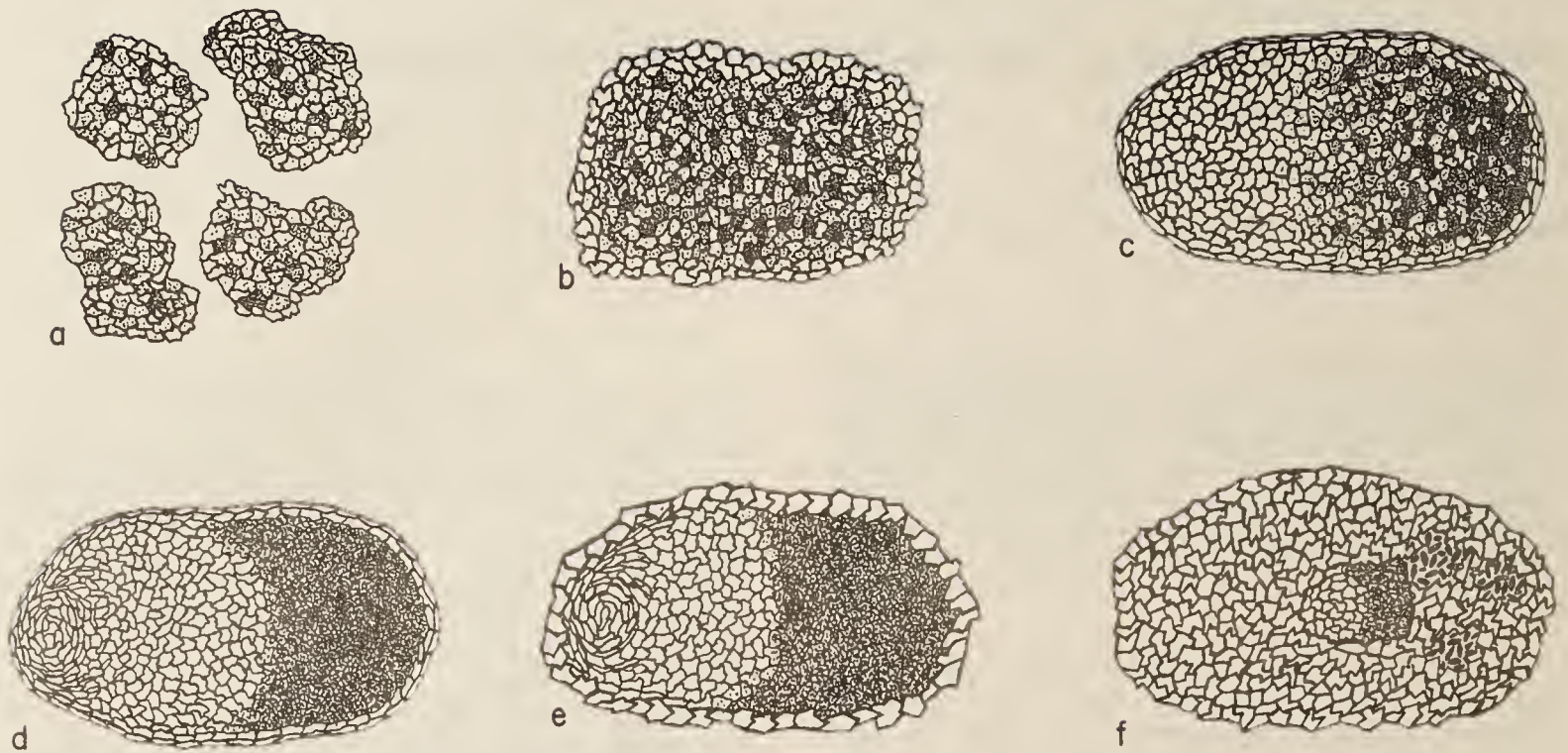


FIG. 1. Diagrams showing the sequence of events of clumps of cells in roller tubes under an atmosphere of oxygen. (a) 2 hr. The cells take up the PAS stain to different degrees, which has been stressed in the diagram. (b) 8 hr. The small clumps have merged into one large clump. (c) 10–12 hr. The prestalk cells form a smooth peripheral ring and the dark staining cells tend to congregate at one end of the clump. (d) 18 hr. There are clear, polar prestalk (left) and prespore zones (right). (e) 22 hr. The stalk cells begin to mature at the outside edge of the clump. (f) 72 hr. Except for the central core of prestalk and prespore cells, all the other cells have turned into either mature stalk cells or spores. This diagram is equivalent to Fig. 2A.

### Evidence that stalk cells and spores are fully differentiated and mature

The evidence comes primarily from electron micrographs (Fig. 2). As can be seen, the stalk cells have characteristically thick walls, large vacuoles, and small residues of cytoplasm including debris and crystals. The spores have thick walls with the appropriately structured layers and a granular internal matrix containing crenulate mitochondria. It is also possible to recognize the characteristic prespore vesicles (6–8). A further test for the presence of mature spores was made by subjecting the clumps of cells to 50° for 30 min, a regimen that kills undifferentiated cells or partially differentiated cells (9). If clumps were first squashed, treated with heat in this manner, and then spread on agar with *E. coli*, amoebae emerged from the spores, proliferated, and gave rise to normal fruiting bodies.

### Conditions affecting differentiation

First it was found that generally a greater number of cells differentiated in the submerged clumps if late interphase cells were used rather than vegetative cells. When such cells were used, if the atmosphere over the salt solution in the roller tubes was flushed daily with pure oxygen (and the oxygen concentration was checked at the conclusion of the experiment with an oxygen tension electrode), the stalk cell differentiation was extensive, while the differentiation of spores was variable, sometimes even absent. No spore or stalk cell differentiation occurred when the tubes were flushed with air. It soon became clear that in order to obtain higher amounts of differentiation, there were other factors in the medium besides oxygen that play a part.

Evidence in support of this possibility comes from experiments on conditioned medium. If cells were cultured in  $10^{-2}$  M salt solution (under an oxygen atmosphere) that has previously contained cells (conditioned medium), then these cells

showed far more spore differentiation. This was consistent in 10 experiments.

It is clear from preliminary studies that a variety of substances affect the degree of differentiation. As Takeuchi has found (personal communication), and we confirm, certain ions have a large effect. The point we wish to emphasize here is that in all the experiments done so far, either with conditioned medium or added substances, the amount of cell differentiation is significantly enhanced by adding oxygen. There are clearly a number of limiting factors, but a high oxygen tension is especially important.

### Regulation of prestalk and prespore regions in clumps

It is well known from the early work of Raper (10) that if a migrating slug is cut into segments, each segment is capable of producing a fruiting body with stalk cells and spores, even though one segment might come exclusively from a prespore (or a prestalk) region. It has been possible to show the same phenomenon with differentiating submerged clumps using neutral red as a means of indicating prestalk and prespore regions. If the clump showed a clear, dark prestalk region and a light prespore region (16–24 hr), the two were separated with a microknife and returned to the salt solution on the roller tube (oxygen added again). After a few hours the portion that was primarily prestalk showed both regions, as did the prespore portion, exactly as had been previously described for migrating slugs (10). This provides further evidence that polar slugs are formed in submerged conditions, as reported earlier (11); this finding had been questioned by Gerisch (12).

### DISCUSSION

It is evident, that contrary to previous views, cell differentiation can occur under submerged conditions. Oxygen appears to be one of the significant limiting factors, although there are others. There are some obvious differences between normal develop-



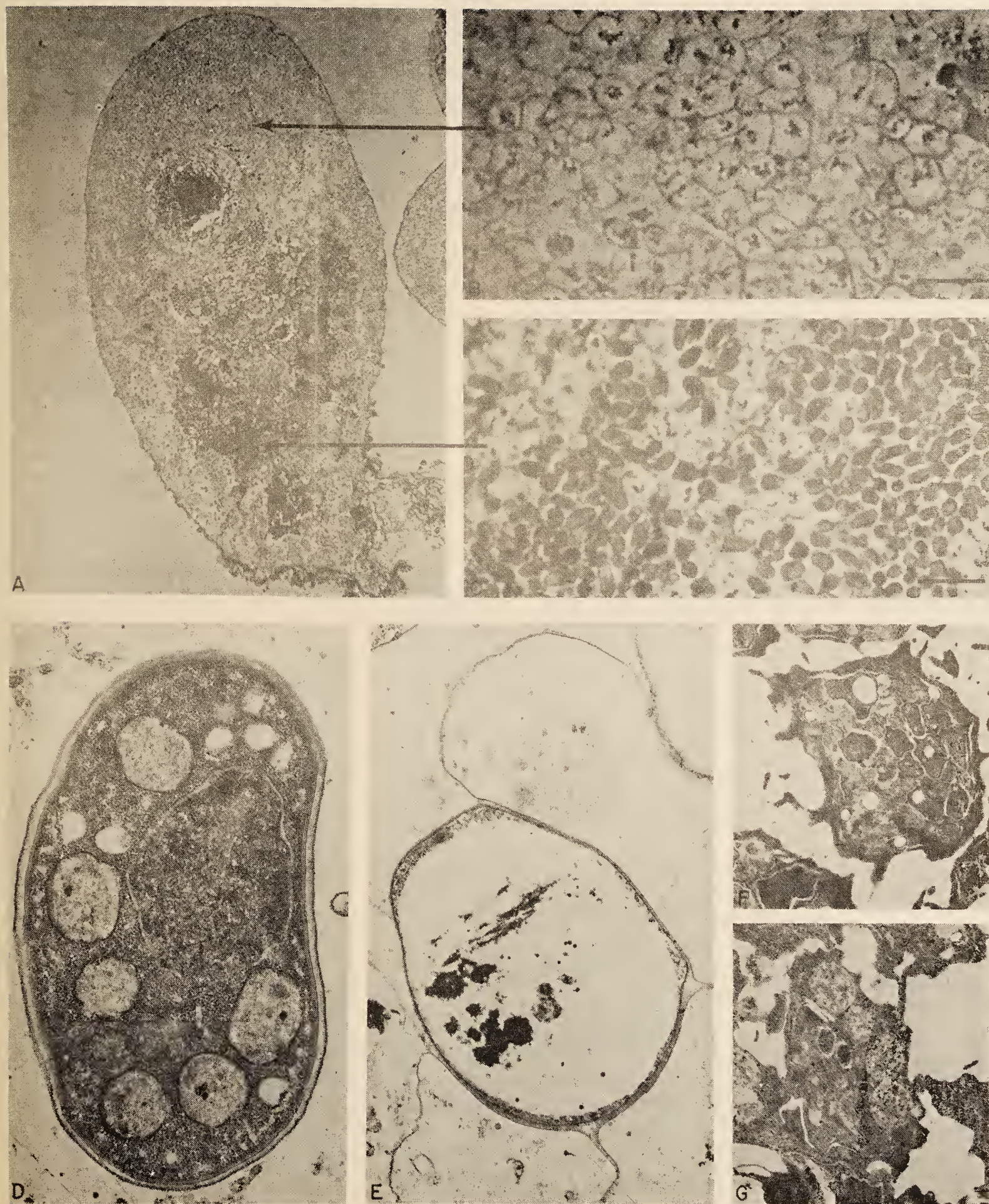


FIG. 2. (A) A longitudinal section of a clump of cells that have been submerged in roller tubes under an  $O_2$  atmosphere for 72 hr. Note that there are mature differentiated stalk cells (shown at higher magnification in B) and differentiated spores (shown at higher magnification in C). The circular dark mass lying above the spores in A is an undifferentiated mass of cells in which prestalk (left) and prespore cells (right) are visible. (D) An electron micrograph of a spore from a clump such as shown in A. (E) An electron micrograph of a mature stalk cell from the same clump. Note the large vacuoles and the thick cell wall. (F) A portion of a prespore cell in which the dark-edged prespore vesicles are clearly visible. (G) A similar view of a portion of a prestalk cell. Note that in these last two electron micrographs the density of the prespore cells is conspicuously greater than that of the prestalk cells. (Magnification: The length of the clump in A is 630  $\mu m$ . The bar in B and C is 10  $\mu m$ . In the remaining photographs the size is given by the width from edge to edge. For D the width is 3  $\mu m$ ; E, 6  $\mu m$ ; F, 4.4  $\mu m$ ; G, 5.3  $\mu m$ .)



ment and that which takes place under water, but the same basic progression of stages still exists. At an early stage the clumps become polarized: a larger number of darkly stained cells are at one end compared to the other. Later, a rigid shell forms around the clump and at the same time the polar distribution of prestalk and prespore cells becomes even more obvious, resembling a normal slug. It is not known by what mechanism this pattern is established.

In order to produce mature stalk cells all that is needed is a rounded mass of cells. It is clear that differentiation can take place without aggregation, but this was known from earlier work as, for instance, in the elegant experiments of Gerisch (12). Also, differentiation can occur without the normal morphogenetic movements associated with culmination. But again this has been shown previously with the fruiting mutant described by Sonneborn *et al.* (13) and for stalk cell induction by cyclic AMP (14, 15). Very recently Town *et al.* (16) have found a mutant of *D. discoideum* that forms both stalk cells and spores without morphogenesis in the presence of cyclic AMP when the cells on the surface of the agar are covered with a layer of cellophane. It is conceivable that at least one factor responsible for the differentiation of these mutant cells is that oxygen can diffuse freely through the overlying cellophane.

With this method of obtaining differentiation in submerged cell masses, the cellular slime molds now provide a system in which it will be possible to do direct biochemical studies not only on cell differentiation, but also on pattern formation.

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## Negative Chemotaxis in Cellular Slime Molds

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This study confirms the suggestion of earlier workers that the vegetative amoebae of *Dictyostelium* repel each other while those of *Polysphondylium violaceum* do not. When *Dictyostelium* amoebae were placed in drops on thin and thick agar, the cells moved out faster on the thin agar, presumably because the repellent was more concentrated. This did not occur with *Polysphondylium* amoebae. Also, if 2 drops of cells were placed side by side, or a single drop was placed near an edge, in *Dictyostelium* there were fewer cells emerging between the drops (or near an edge) than on the far side. *Polysphondylium* showed no such difference. However, *Polysphondylium* amoebae were repelled by *Dictyostelium* cells (but not vice versa) when drops of each were placed beside one another. Finally, if *Dictyostelium discoideum* cells were placed in drops over thick and thin agar, but separated from the agar by a dialysis membrane, the cells again spread farther on the thin agar, indicating that the repellent is a dialyzable molecule.

The first evidence for negative chemotaxis came from the work of Samuel (5), who showed in *Dictyostelium discoideum* that vegetative or preaggregation cells tend to repel one another. He placed a concentrated drop of amoebae on non-nutrient agar and put a small cellophane square (covered with a thin layer of agar) near it. When a few of the cells escaping from the concentrated drop moved onto the cellophane, he turned the square 90°. Twenty minutes later, the cells made a right-angle turn and again moved away from the drop of cells. Another way of testing for negative chemotaxis was suggested by Lee Segal (personal communication): if the cells in a drop repel each other, the rate at which they move away should be linear with time, whereas if they leave the drop as a result of random motion, then their speed of outward movement should fall off as the square root of time. Adam (senior thesis, Princeton University, Princeton, N. J., 1973) showed that, indeed, preaggregation amoebae of *D. discoideum* did seem to spread linearly with time, but he also made the interesting observation that in another species, *Polysphondylium violaceum*, the cells move outward in a manner that appeared very roughly equivalent to the square root of time. If these preliminary observations are correct, they would suggest that *D. discoideum* has a repellent and *P. violaceum* does not. The present paper gives evidence that strongly supports such a contention.

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### MATERIALS AND METHODS

The cellular slime molds used were: (i) *D. discoideum* NC-4H; (ii) *D. mucoroides* no. 11; *D. purpureum* no. 2; and *P. violaceum* no. 1. They were all grown on *Escherichia coli* B/r.

The slime molds were incubated on nutrient agar (buffered 1% peptone and glucose agar) for approximately 40 h at 21°C in the light. They were then harvested and centrifuged three times for 7 min each at  $75 \times g$  in 1% ( $10^{-4}$  M) standard salt solution (1). The washed amoebae were then suspended in 1% salt solution at a concentration of  $10^8$  cells per ml. Most of the experiments were run on 2% non-nutrient agar (Difco) in small plastic petri dishes (50 by 12 mm; Falcon no. 1006).

### RESULTS

**Experiments with deep and shallow agar.** The design of experiments with deep and shallow agar followed the principle of those of Twitty and Niu (reviewed by Twitty, 6) for pigment cells in amphibians. Agar was poured into the petri dishes at two depths, 0.5 and 4 mm. Drops of washed amoebae were placed on the surface, and measurements were made of the distance travelled by the outermost cells after 2 and 4 h (see reference 4). *D. discoideum* amoebae moved out much further on the shallow agar than on the deep agar, which is what one would expect if a repellent were responsible for the outward movement of the amoebae (Fig. 1A, Table 1). The same conclusion can be reached for *D. purpureum*. The result is ambiguous for *D. mucoroides* and clearly reversed for *P. violaceum*, in which greater spreading oc-

curs on the deep agar. In this case one might postulate that an attractant plays a part and that since it is more concentrated in the shallow agar, the cells are attracted inward, countering the effect of the outward movement of the cells by random motion. Because the air space was different in the small petri dishes with the two

depths of agar, a control with *P. violaceum* was run in which open petri dishes were placed in a moist chamber. The results (for 24 drops) were similar to those described in Table 1; clearly, there is no volatile component affecting the outward movement of the cells.

**Experiments with 2 drops.** In this sort of experiment, 2 drops were placed close to one another, and after approximately 2 h the number of cells seen through an ocular grid on the dissecting microscope (area, 0.86 mm<sup>2</sup>) was counted in two places: on an edge close to the other drop of cells (inside) and on an edge farthest removed from the other drop (outside). If the drops are separated by a range of distances between 1.4 and 1.9 mm, then, as before, the *D. discoideum* amoebae appear to repel one another, whereas the *P. violaceum* amoebae do not (Fig. 1B, Table 2). When the drops are too close (<1 mm apart), then the cells from both drops mingle, making it impossible to record cell density in a meaningful fashion.

This kind of experiment was repeated with one major difference; a drop of *P. violaceum* cells was placed next to a drop of *D. discoideum* cells. It is clear that *P. violaceum* amoebae are effectively repelled by the *D. discoideum* drop but that the *P. violaceum* drop does not repel the *D. discoideum* amoebae (Table 3).

**Experiments with cell drops near an agar edge.** This is essentially the same experiment as described above, but instead of confronting 1 drop of cells with another, the agar is cut off sharply near the edge of a drop and kept in a moist environment. If the repellent is not vola-

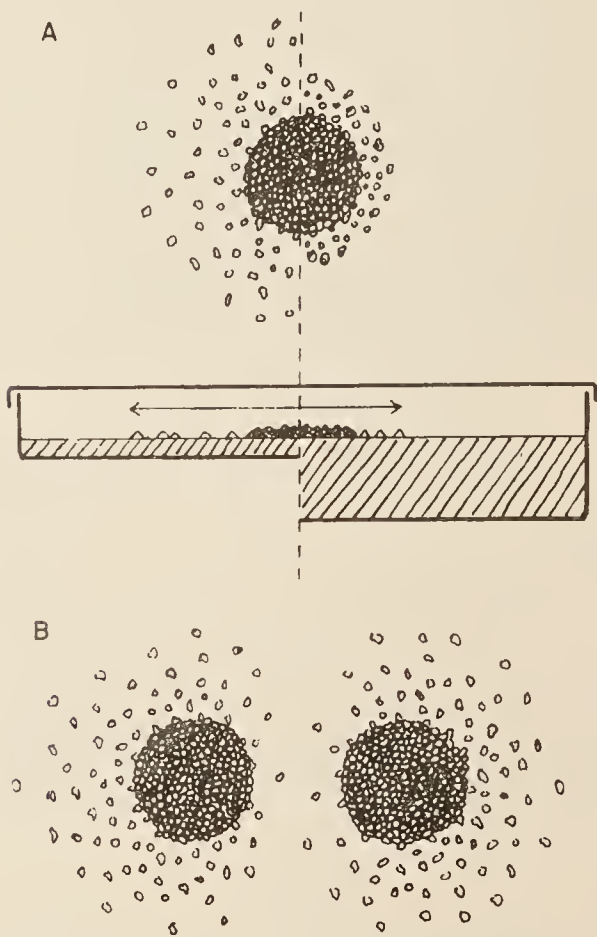


FIG. 1. Diagrams showing the two basic types of experiments used to demonstrate negative chemotaxis. (A) Thin versus thick-agar experiment seen from surface view (above) and side view (below). Note that the cells spread farther on the thin agar. (B) Two drops of *D. discoideum* cells side by side on an agar surface. Note that the cell density between the drops is lower than in any other position.

TABLE 2. Number of cells spread between 2 drops (inside) compared to the number of cells spreading where there are no opposing drops (outside)<sup>a</sup>

Species	Mean no. of cells/mm <sup>2</sup> (± SD)		No. of cases	Probability (2-tailed <i>t</i> test)
	Outside	Inside		
<i>D. discoideum</i>	106 (±17)	83 (±20)	18	0.001 (3.614)
<i>P. violaceum</i>	98 (±79)	100 (±72)	26	>>0.1 (0.078)

<sup>a</sup> The distance between the drops is in the range of 1.4 to 1.9 mm. SD, Standard deviation.

TABLE 1. Rates of spreading of cells on shallow and deep agar<sup>a</sup>

Species	Rate of spreading (mm/h ± SD) in:		No. of cases	Probability (2-tailed <i>t</i> test)
	Shallow agar	Deep agar		
<i>D. discoideum</i>	0.42 (±0.19)	0.29 (±0.14)	38	0.001 (4.889)
<i>D. purpureum</i>	0.36 (±0.15)	0.20 (±0.07)	31	0.001 (5.237)
<i>D. mucoroides</i>	0.20 (±0.09)	0.15 (±0.08)	24	0.05 (1.889)
<i>P. violaceum</i>	0.44 (±0.13)	0.54 (±0.14)	36	0.001 (3.259)

<sup>a</sup> These tests were each repeated for a second reading giving similar results except for *D. mucoroides*, which showed identical spreading in both conditions. SD, Standard deviation.



TABLE 3. Number of cells spread between (inside) a drop of *D. discoideum* and a drop of *P. violaceum* (1.4 to 1.9 mm apart) compared to the number of cells that spread where there are no opposing drops

Species	Mean no. of cells/ mm <sup>2</sup> ( $\pm$ SD) <sup>a</sup>		No. of cases	Probability (2-tailed <i>t</i> test)
	Outside	Inside		
<i>D. discoideum</i>	78 ( $\pm$ 16)	85 ( $\pm$ 12)	11	>0.1 (1.247)
<i>P. violaceum</i>	91 ( $\pm$ 25)	37 ( $\pm$ 19)	11	0.001 (5.678)

<sup>a</sup> SD, Standard deviation.

tile, as previously described experiments indicated, then it should accumulate in greater concentration near an edge than on the opposite side, where there is a long stretch of agar for continued diffusion. Here, over a wide range of distances (1.4 to 2.5 mm) between the drop and the edge, it is clear that in *D. discoideum* there are fewer cells on the edge side (hence a greater concentration of repellent), whereas there is no difference between sides for *P. violaceum* (hence no repellent) (Table 4).

Experiments with a dialysis membrane. To see if the repellent will pass through a dialysis membrane, the experiment with shallow and deep agar was repeated, but in this case the agar was covered with a dialysis membrane (which had been washed by boiling for 10 min in 10<sup>-3</sup> M sodium ethylenediaminetetraacetate). To keep the membrane flat, it was placed in a small double-ring frame resembling a miniature embroidery hoop. The depths of the agar were 0.3 mm (shallow) and 11 mm (deep) in plastic petri dishes (100 by 15 mm; Falcon no. 1001).

The results are similar to the previous shallow-deep experiments (Table 1): the amoebae of *D. discoideum* spread much farther on the shallow dishes, 0.37 ( $\pm$ 0.06) mm/h, than on the deep dishes, 0.27 ( $\pm$ 0.07) mm/h, in 14 and 16 cases tested ( $P = 0.001$ ; 3.653 by two-tailed *t* test). Therefore, the repellent is a small molecule that can pass through a dialysis membrane. We are presently attempting to devise a bioassay so that further characterization of the repellent will be possible.

## DISCUSSION

It is clear from these experiments that the vegetative amoebae of *D. discoideum* (and *D. purpureum*) produce a dialyzable repellent that causes the amoebae to spread away from one another. The situation for *D. mucoroides* is marginal, but *P. violaceum* does not produce the repellent, although it is sensitive to that produced by *D. discoideum*.

TABLE 4. Number of cells spread from a drop near an agar edge (edge side) compared to the number of cells that spread on the side where there is extended agar (outside)

Species	Mean no. of cells/ mm <sup>2</sup> ( $\pm$ SD)		No. of cases	Probability (2-tailed <i>t</i> test)
	Outside	Edge side		
<i>D. discoideum</i>	63 ( $\pm$ 13)	46 ( $\pm$ 9)	12	0.001 (3.740)
<i>P. violaceum</i>	220 ( $\pm$ 28)	229 ( $\pm$ 37)	6	>>0.1 (0.459)

<sup>a</sup> The distances between the drop and the edge range from 1.4 to 1.9 mm. SD, Standard deviation.

It is unlikely that any of the differences shown here are due to differences in rates of locomotion of cells, but rather, they are due to differences in orientation. This was shown by Bonner et al. (3) and Adam (senior thesis), for the rates of movement of individual amoebae are not significantly affected by gradients of acrasins; they only affect cell orientation.

Another basic difference between *Dictyostelium* and *Polysphondylium* is that the former uses cyclic adenosine 3',5'-monophosphate for its acrasin, whereas the latter does not. During the vegetative stages, it is known that the *Dictyostelium* amoebae produce a small amount of extracellular cyclic adenosine 3',5'-monophosphate and have some sensitivity to its gradients at that stage (2). Therefore, the repellent must overcome or counteract any tendency for the vegetative cells to clump; and it can only be when the cyclic adenosine 3',5'-monophosphate production and the sensitivity to it are each increased dramatically that the repellent can be counteracted and aggregation can occur. On the other hand, in *Polysphondylium* we have presented evidence that the attractant is operating at all stages; it is simply not strong enough to cause genuine aggregation at the vegetative stage.

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## COMPLEMENTARY EFFECTS OF AMMONIA AND cAMP ON AGGREGATION TERRITORY SIZE IN THE CELLULAR SLIME MOLD *DICTYOSTELIUM MUCOROIDES*

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### SUMMARY

Ammonia is known to increase and cAMP to decrease the size of aggregation territories in *Dictyostelium mucoroides*. Here we show that cAMP will counteract the effect of ammonia, and ammonia will cause a decrease in the secretion of cAMP.

Control mechanisms in development often consist of pairs of substances which have opposite effects; one acts as a stimulator and the other as an inhibitor of some process. Here some evidence is presented to suggest that cyclic AMP (cAMP) and ammonia act in such a complementary fashion in the development of a cellular slime mold.

It has been known for some time that *Dictyostelium mucoroides* produces extracellular cAMP which acts as a chemoattractant or acrasin during the aggregation phase (review [13]) and that it also could possibly be the normal agent that stimulates the formation of stalk cells since it is capable of inducing stalk cell formation in isolated cells [2, 6, 9]. Finally, it is known to affect territory size: the higher the concentration of extracellular cAMP, the more aggregation centers per cm<sup>2</sup> on the surface of the substratum [3, 13].

It has been shown by Cohen [7], Gregg et al. [12], and Lonski [14], that cellular slime molds give off ammonia, and Feit [8] and Lonski [14] have evidence that ammonia plays an important role in the aggregation territory size: the higher the ammonia concentration, the fewer the number of centers per cm<sup>2</sup>. As will be shown, not only are these two substances complementary in their effects on territory size, but the production of extracellular cAMP can be directly inhibited by increasing the concentration of ammonia.

### MATERIALS AND METHODS

For the majority of the experiments *Dictyostelium mucoroides* (strain no. 11) was used. Some comparison experiments were done with *Polysphondylium violaceum* (strain no. 1). The amoebae were grown in a two-membered culture with *Escherichia coli* (strain B/r) for 40 h at 21°C in the light on 1% peptone-dextrose (buffered) agar. The cells were washed free from the surface of the plates and centrifuged at 75 g three times for 7 min each with 1% (10<sup>-4</sup> M) standard salt solution [1].

In the experiments on territory size, the washed

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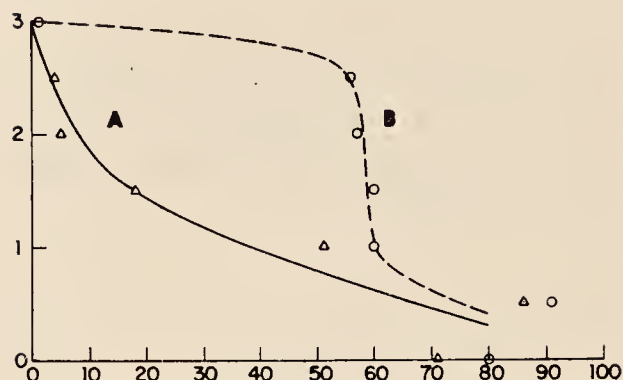


Fig. 1. Abscissa: aggregation centers/cm<sup>2</sup>; ordinate: NH<sub>4</sub>Cl (mM).

The effects of varying concentrations of ammonia (A) and the compensating effects of added cAMP (B) on the aggregation territory size of *D. mucoroides*. The cells are at a density of 400 cells/mm<sup>2</sup>. Each point is an average of twelve measurements of aggregation center density.

cells were suspended in 50% ( $5 \times 10^{-3}$  M) standard salt solution and diluted so that the ultimate concentration on the agar surface was 400 cells/cm<sup>2</sup>. The cells in the salt solution were allowed to settle on 2% non-nutrient agar in small Petri dishes (bottoms only; 60 × 15 mm, Falcon no. 1007) for 30 min and then the excess fluid was pipetted off. The agar plates were inverted over Syracuse dishes (68 × 17 mm) which contained a mixture of 1 ml of 1 N NaOH and varying concentrations of NH<sub>4</sub>Cl. The two dishes were sealed together with masking tape and incubated at 23°C in the light. The number of aggregation centers per unit area was counted after 6 and 24 h, by averaging the number of centers in six fields taken at random through the dissecting microscope and converting to centers/cm<sup>2</sup>.

To measure the amount of cAMP given off by the vegetative and aggregating cells, the "drum" technique described previously [3] was used to collect the cAMP. It consists of two close-fitting plastic cylinders (80 × 53 mm) which interlock to hold a taut sheet of dialysis membrane. The drum is set over 50 ml of distilled water in a crystallizing dish (92 × 22 mm) so that the bottom surface of the membrane is in contact with the water. In this way cAMP can diffuse through the membrane into the water, while the phosphodiesterase which inactivates it cannot. The *D. mucoroides* cells were allowed to settle on the upper surface of the dialysis membrane in a concentration of 2000 cells/mm<sup>2</sup> and, as above, the excess water was pipetted off. Three such drums were placed in large boxes (100 × 260 mm) with close-fitting lids. One set also had a Petri dish containing the ammonia generating solutions described previously. Every 2.5 h for 10 h, and once more after 24 h, the water under the dialysis membrane was removed and replaced with fresh distilled water.

The cAMP in the removed water was assayed using the methods of Gilman [11] and Brostrom & Kon [5].

## RESULTS

### Effect of ammonia and cAMP on territory size

The earlier observation of Feit [8] and Lonski [14] that NH<sub>3</sub> causes an increase in territory size (fewer centers/cm<sup>2</sup>) was repeated for *D. mucoroides* at a concentration of 400 cells/mm<sup>2</sup> on 2% non-nutrient agar exposed to atmospheres of different concentrations of ammonia (fig. 1, A). If, in some of the plates, 5 μl of 10<sup>-4</sup> M cAMP was spread over the surface just before their exposure to ammonia, the effect of ammonia is to a large extent counteracted (fig. 1, B). This experiment was repeated three times with the same result. Since it is known that high concentrations of cAMP alone will decrease the territory size [3, 13], it is evident that not only do these two substances have opposite effects on territory size, but one will counteract the action of the other.

These experiments were repeated on *Polysphondylium violaceum*, a species that

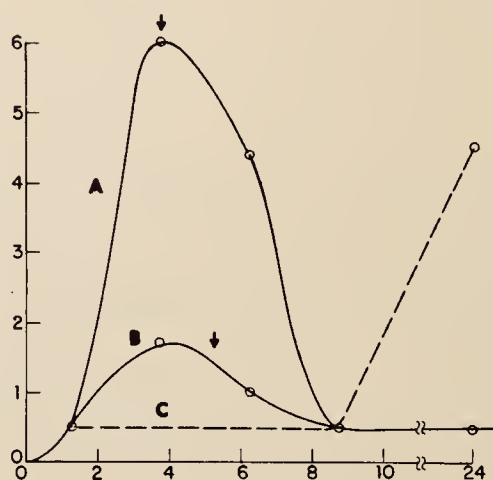


Fig. 2. Abscissa: time (hours); ordinate: cAMP (pmoles/μl).

The quantities of cAMP produced during the course of aggregation of *D. mucoroides* without (A) and in the presence of two different concentrations of ammonia (B, C). In (C) the ammonia was removed at 10 h in some of the dishes. The time of aggregation is indicated by a vertical arrow.



does not use cAMP as its acrasin [16], and while ammonia had the same effect on territory size, it was not counteracted by cAMP. It was also shown that not only did ammonia delay the onset of aggregation for both *D. mucoroides* and *P. violaceum*, but this time interval is not affected by varying concentrations of cAMP with or without ammonia in either species.

#### *Effect of ammonia on extracellular cAMP production*

When the amoebae of *D. mucoroides* were placed on the dialysis membrane of a drum at a density of 2000 cells/mm<sup>2</sup>, the liquid below contained the secreted cAMP of the cells that had escaped enzymatic conversion to 5'AMP. In this case the liquid from three drums was harvested every 2.5 h, and the cAMP content determined by the Gilman assay. As can be seen in fig. 2, A, in the absence of added ammonia the cAMP rose to a sharp peak, as reported previously for *D. discoideum* [3, 15]. At the same time another three drums were incubated in a plastic box similar to the one in the controls, but containing 5 ml of 1 N NaOH and 5 ml of 0.015 N NH<sub>4</sub>Cl. This quantity was determined by trial and error; it produces enough ammonia to inhibit, but not enough to completely block aggregation. In this particular experiment the amount of ammonia added is unknown, but in general the amounts added are within the range of what the amoebae normally give off [14]. As can be seen from fig. 2, B, the ammonia not only delays aggregation, but clearly reduces the amount of cAMP produced. This same experiment was repeated three times with essentially the same results although the absolute values varied slightly.

In another experiment with three drums at the same cell density, the cells were exposed to the ammonia generated from 5 ml

of 1 N NaOH and 5 ml of 0.03 N NH<sub>4</sub>Cl. This amount completely blocked aggregation and there was no cAMP production. However, if after 10 h the ammonia is removed, the amoebae then aggregate and the aggregation is accompanied by the usual burst of cAMP production (fig. 1, C). This experiment was repeated three times with the same results.

## DISCUSSION

It was shown in an earlier study [4] that some species were sensitive to a volatile "spacing substance" which causes larger territories (e.g., *P. violaceum*, *P. pallidum*, and *D. mucoroides*) while others were not (e.g., *D. discoideum* and *D. purpureum*). As pointed out above, various authors [8, 14] have evidence that this volatile agent which increases territory size is ammonia since it is produced by the cells in amounts which are known to limit the territory size.

It is also known that adding excess cAMP to the agar results in a reduction in the territory size [3, 13]. According to the work of Westra [cited in 13] this apparently only occurs in species that are sensitive to cAMP (e.g., all large *Dictyostelium* species and no species of *Polysphondylium*).

Here we have shown that cAMP counteracts the ammonia effect on territory size in *D. mucoroides* and that ammonia reduces the extracellular production (and therefore possibly the synthesis) of cAMP. The two substances seem to play complementary roles and ammonia does this by limiting the production of cAMP. There is no evidence on how cAMP affects ammonia production; it may not affect it at all and could act by directly influencing the ability of the cells to respond to ammonia.

It is worth pointing out that while ammonia affects the territory size of both *D.*

*mucoroides* and *P. violaceum*, cAMP only affects the former, and that it is only in this species that cAMP can be used to counter the effect of ammonia. It will be important, when *P. violaceum* acrasin has been isolated and identified, to see if its production is also limited by these physiological levels of ammonia.

It would be of particular interest to know if there are other processes besides territory size that are controlled by the stimulation and inhibition of these two substances. For instance cAMP is possibly involved in cell differentiation; is this true of ammonia as well? Many models of developmental control require the interaction between an activator and an inhibitor substance. A good example is the reaction-diffusion model developed by Geirer & Meinhardt [10] where not only are two such substances required, but the inhibitor must be a smaller molecule than the activator, conditions which are met by ammonia and cAMP. But clearly it is not yet time to speculate which models most appropriately describe slime mold development: we want to know more about the physiological activities of ammonia and cAMP.

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## The Prestalk-Prespore Pattern in Cellular Slime Molds

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In cellular slime molds the slugs become divided into two regions with different properties, an anterior *prestalk zone* and a posterior *prespore zone*. Although the cells in these zones are normally destined to form the stalk cells and spores of the fruiting body, respectively, they are not irreversibly committed to one sort of differentiation or the other during the slug stage. The volume ratio of the two zones remains almost constant over a wide range of slug sizes. If the prestalk-prespore pattern is disturbed by removing tissue from the slug, conversion of tissue from prestalk to prespore or vice versa occurs as necessary to restore a normal pattern with normal proportions. Conversions also occur in both directions during normal development. The initial formation of the prestalk-prespore pattern may well involve sorting-out, but other mechanisms must be invoked to account for regulation.

We describe three different models of the generation of the prestalk-prespore pattern, the 'cell-contact model' of McMahon, in which pattern is created by interactions of cells with their immediate neighbors, the 'positional-information model' of various authors, in which pattern formation involves an overall gradient and a gradient-reading mechanism, and the 'activator-inhibitor model' of Gierer and Meinhardt, in which the prestalk-prespore pattern is formed by a system of diffusible substances that affect one another's production. The activator-inhibitor model is the most successful of the models at describing the known features of the prestalk-prespore pattern.

The various models lead to a number of distinctive predictions. According to the cell-contact model, small transplants may cause gross changes in the prestalk-prespore pattern, and mutants may exist which severely disrupt pattern formation even if diluted with a large excess of wild-type cells. Positional-information models predict the existence of 'gradient-reading mutants'; slugs that are a mixture of such mutants and wild-type cells would show two prestalk-prespore boundaries, one at the mutant and one at the normal position. Both the activator-inhibitor model and some versions of the positional-information model predict that small transplants will sometimes induce accessory prestalk or prespore zones; the quantitative characteristics of these effects may allow one to make a case in favor of one or other of the two models. Finally, the activator-inhibitor model leads one to expect that mutants may be isolated which normally show accessory prestalk or prespore zones. A search for these phenomena may help determine whether the activator-inhibitor model will continue to enjoy its present preeminent position.

### Introduction

In the cellular slime molds the amoebae feed and grow as separate cells; they then aggregate into cell masses, which ultimately form fruiting bodies consisting of stalk

cells and spores. Under certain conditions the cell mass which forms from aggregating amoebae migrates across the substratum before forming a fruiting body. Using vital dyes and various other techniques, two zones can be demonstrated in this migrating 'slug': the anterior



'presumptive stalk zone' (consisting of *prestalk* cells) and the posterior 'presumptive spore zone' (*prespores*) [1, 2]. Cells of these two zones are normally destined to become the stalk cells and the spores of the fruiting body, respectively. We do not now understand how the *prestalk-prespore pattern* is formed, but the problem is intriguing and may be of general significance, since the mechanism involved may be similar to the mechanisms responsible for pattern formation in other multicellular organisms.

In this paper we will discuss what is now known about the formation and regulation of the prestalk-prespore pattern. We will begin by reviewing what is known about the pattern itself, and will next consider other properties of the slime mold slug which may be relevant to the pattern formation problem. We will finally examine three theoretical models, the 'cell-contact model', the 'positional information model', and the 'activator-inhibitor model', each of which might be a useful description of pattern formation in the cellular slime molds.

### Characteristics of the Prespore-Prestalk Pattern

#### *Differences Between Prestalk and Prespore Cells*

The prestalk-prespore pattern was first demonstrated with vital dyes [2]: neutral red, Nile blue, and bismarck brown were all found to stain an anterior zone, generally 15 to 30% of the slug, more intensely than the rest (Fig. 1). This differential staining has never been completely explained, but it seems likely that autophagic vacuoles in the prestalk cells play an important role, at least in the case of neutral red. The deep color of neutral-red stained prestalk tissue seems, on microscopic examination, to be due to intense staining of 'granules' which resemble the autophagic vacuoles described by Maeda and Takeuchi [3] in both size and abundance (Fig. 2). These granules are found predominantly in the anterior part of the slug. A number of other cases are known in which neutral-red staining granules have been identified as vesicles of the lysosomal system [4-7].

Early histochemical studies showed that the prespore zone stains intensely with the PAS (periodic-acid-Schiff) procedure (for non-starch polysaccharides) while the prestalk zone shows strong alkaline phosphatase, cytochrome oxidase, and succinic dehydrogenase activities [1, 8, 9]. Enzyme activity differences between the prestalk and prespore zones have been demonstrated using direct methods by Newell and coworkers [10], who found uridine diphospho (UDP) galactose : polysaccharide transferase only in the prespore zone, and by Rutherford and coworkers [11-13] who found that glu-



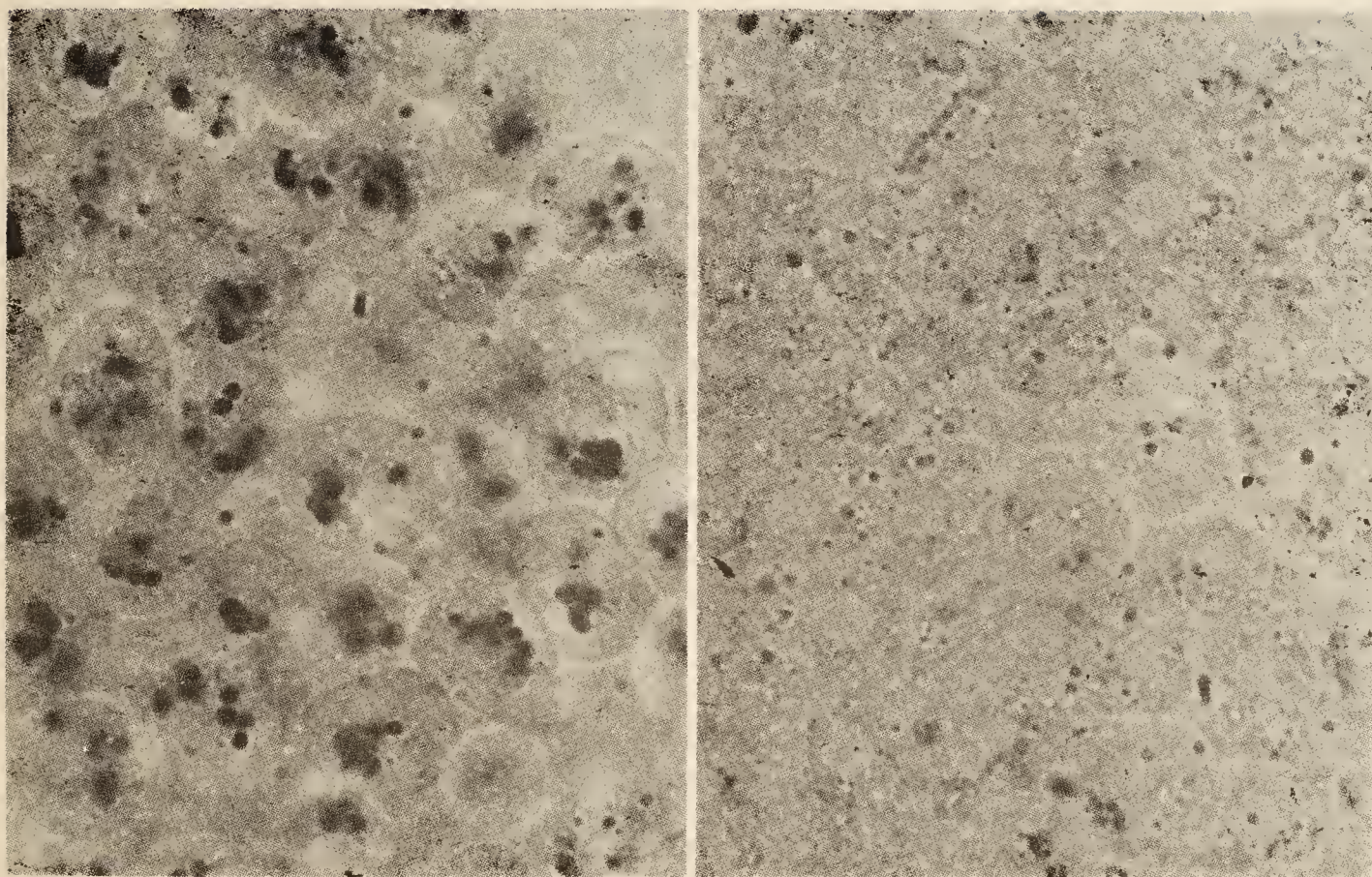
Fig. 1. The prestalk-prespore pattern in *D. discoideum* (strain NC-4) as revealed by vital staining.  $2 \times 10^8$  preaggregation amoebae were suspended in 1 ml of a solution of neutral red which had an O.D.<sub>550</sub> of 2.0. The amoebae were immediately spun down and the pellet dispensed in drops of about  $10^7$  cells on 1.5% agar in 35 mm petri plates

cose, trehalose, trehalase, and glycogen phosphorylase all have significantly higher levels in prestalk tissue than in prespore tissue.

Prestalk cells are a few percent denser than prespores; this allows dissociated slugs to be separated into the two cell types by centrifugation. Preparations of prestalk and prespore cells obtained with such methods have been found to differ in the activities of alkaline phosphatase, beta-glucosidase, and beta-galactosidase, all of which are higher in the prestalk zone, and in UDP glucose : pyrophosphorylase, malate dehydrogenase, citrate synthetase, and UDP galactose : polysaccharide transferase, which show higher activities in prespores. Acetylglucosaminidase activity is reported to be higher in prestalk cells of *Dictyostelium mucoroides*, but higher in the prespores of *Dictyostelium discoideum* [14, 15]. Acid phosphatase and beta-galactosidase are both reported to have isozymes which appear to be confined to the prestalk zone [14].

Alton and Brenner [16] have used two-dimensional gel electrophoresis to demonstrate a number of differences in the protein synthesis patterns of prestalk and prespore tissue. Gregg and Karp [17] have shown that prespore cells incorporate fucose, while prestalk cells do





**Fig. 2.** Vital staining of cells from *D. discoideum* slugs. Fragments of prestalk (*left*) or prespore (*right*) tissue from neutral-red-stained slugs were gently squashed in a drop of water on a microscope slide. The preparation reveals intensely-staining 'granules' in the prestalk cells which may be autophagic vacuoles. Few of the granules are seen in prespore cells

not, a feature which allows the prestalk-prespore pattern to be visualized in autoradiograms.

A particularly effective method of demonstrating the prestalk and prespore regions was devised by Takeuchi [18] who obtained an antibody against spores and conjugated this with a fluorescent molecule. Sections of slugs stained with the labeled antiserum showed the spore antigen confined to the posterior prespore region.

Electron microscopists have found that only the prespore cells have 'prespore vesicles' which presumably contain the antigenic material of the spores [3, 19–23, but see 24]. Both prestalk and prespore cells contain autophagic vacuoles [3] in which acid phosphatase [25] has been demonstrated; these vacuoles are considerably larger and more numerous in the prestalk cells. Prestalk cells are in closer contact with one another than prespores, and have thinner membranes [3]. The cells of the prestalk and prespore zones also differ in their adhesiveness to plastic [26] and in their size [27].

A number of the differences between prestalk and prespore tissue anticipate differences between differentiating stalk cells and spores. It thus seems plausible to interpret the formation of the prestalk-prespore pattern

as an initial step along the pathway to the final differentiation of stalk cells and spores. The parallels supporting this interpretation include:

1. Stalk cells become extensively vacuolated during final differentiation; this has been attributed to further enlargement of the hypertrophied autophagic vacuoles found in prestalk cells [3].

2. Alkaline phosphatase, glycogen phosphorylase, and trehalase, all of which have higher activities in the prestalk zone than among prespores, all appear to play roles in stalk differentiation, while they are unimportant in spores [11, 13, 28, 29].

3. UDP galactose : polysaccharide transferase, which accumulates in the prespore zone but not in the prestalk zone of the slug, is restricted in the fruiting body to spores [10].

4. The presence of a spore antigen exclusively in prespore cells [18] also suggests predifferentiation.

### Regulation

It has been recognized since the early experiments of Kenneth Raper [30] that cells from the posterior end of



a migrating slug, all of which would normally produce spores, will, when isolated, produce a normal fruiting body containing both stalk cells and spores. Similarly, cells from the anterior end of the slug, which would normally produce stalk cells, will produce both stalk cells and spores if the posterior is removed.

When parts of the slug are removed, regulation soon occurs in the prestalk-prespore pattern. This regulation was first demonstrated using histochemical methods [1]. More recently, Gregg [31] studied this regulation using immunofluorescent techniques and found that prestalk cells undergoing conversion to prespore revert to the synthesis of an early antigen; this does not appear to be true of prespore cells becoming prestalk. Sakai has demonstrated that regulation in both directions (as judged by the appearance or disappearance of prespore vesicles) requires RNA and protein synthesis [32].

Regulation occurs not only when a slug is fragmented but also during normal development. *D. mucoroides*, which is capable of extended migration, produces stalk continuously while migrating. The result is a constant depletion of the prestalk cells, yet the proportion of prestalk to prespore volumes in the undifferentiated slug tissue remains allometric (see below) over long periods of migration, which can only mean there is a continuous conversion of prespore to prestalk cells [33]. Recently, Hayashi and Takeuchi [34] have demonstrated a similar phenomenon in *D. discoideum* using Takeuchi's [18] fluorescent labeled spore antiserum described above. They showed that the ratio of cells with and without fluorescence is maintained even as the mature stalk is being formed at the expense of prestalk cells, again implying that prespore cells acquire prestalk character during normal development. It is also worth noting that before culmination is complete, a substantial number of prestalk cells must become spores (see *Quantitative Characteristics of the Prestalk-Prespore Pattern*, below).

The existence of prestalk-prespore interconversion has been taken to imply that the prestalk and prespore cells are not predifferentiated in any meaningful sense, and that 'the pattern of differentiation is specified during culmination' [35]. We do not agree with this assessment. As indicated above, we feel that many of the features of prestalk-prespore pattern in fact represent the beginnings of stalk and spore differentiation. It also seems significant that the prestalk-prespore ratio changes in response to a variety of treatments in a manner similar to the ratio of stalk cells to spores (see *Factors Which Shift the Prestalk : Prespore Ratio*, below). The fact that prestalk and prespore cells in the slug are not irreversibly committed to stalk cell or spore formation

only shows that differentiation in cellular slime mold slugs can proceed to a certain extent in the absence of determination. Biologists may be accustomed to think of determination as preceding differentiation, but we know no a priori reason to suppose that this sequence of events is obligatory; cellular slime molds seem to provide a counterexample.

There is in fact evidence that prespore and prestalk cells are 'somewhat' committed to stalk cell and spore differentiation. Raper showed that regulation is quite slow in the prestalk region: Amputated anterior portions of a slug produce 'stalky' fruiting bodies if they are caused to culminate within six hours after isolation [30]. Sampson showed that Raper's results are also obtained if the isolated fragments are disaggregated before they are allowed to fruit [36]. The stalkiness of anterior fragments thus reflects some characteristic of the prestalk cells themselves rather than extracellular factors. Sampson also showed that if a slug is allowed to migrate for a time before being cut up, the proportion of spores in fruiting bodies formed by posterior fragments increases. There is, in other words, a tendency of posterior cells to form spores which increases with age of the slug. Neither this tendency nor the stalky tendency of anterior cells is irreversible, as the regulation experiments show, but both can be measured under appropriate experimental conditions.

#### *Mode of Formation and Regulation of the Prestalk-Prespore Pattern*

When early aggregates are stained with fluorescent anti-spore serum, they appear to contain a random mixture of staining and nonstaining cells [18]. This then converts into a two-zoned pattern [18, 37, 38] in a process which is generally complete before migrating slugs are formed. The conversion process has been interpreted as sorting-out [18], although the role of sorting has not been conclusively established. An appearance of sorting has also been reported in the development of the prestalk-prespore pattern in submerged aggregates [39, 40]; this is disputed in other studies [38]. Cell sorting is described further below.

In the regeneration of a prestalk or prespore zone it seems most likely that sorting-out is *not* involved. The new zone seems instead to be formed by conversion of prespore cells to prestalk, or vice versa, the conversion beginning at one end of the slug and propagating in a wavelike fashion until the normal prestalk : prespore proportion is reestablished [1; see also 17].



During regulation, the formation of a new prespore or prestalk zone, monitored by PAS staining [1] takes several hours. Prespore vesicles take several hours to appear or disappear [32] and the 'stalkiness' of prestalk fragments persists several hours after isolation (see above). However, Gregg and Karp have presented evidence, obtained by monitoring the characteristic fucose incorporation by prespore cells, that a new prespore zone can be formed in significantly less than fifteen minutes [17]. A plausible way to account for these differences is to suppose that most of the properties which distinguish prestalk from prespore tissue are the results of some sort of cell differentiation, and that time is required to complete this differentiation, but a 'primary' prestalk-prespore pattern exists that is somewhat more labile.

#### Quantitative Characteristics of the Prestalk-Prespore Pattern

Raper noted in his original description of *Dictyostelium discoideum* that the proportions of the fruiting body remain approximately constant regardless of its size [41, 42]. A roughly constant stalk-spore proportion has since been demonstrated in *D. discoideum* using linear measurements of stalk height and sorus radius [43, 44], measurements of stalk and sorus volumes [33, 44], and dry weights [2]. More recent investigations suggest, however, that the constancy of proportions is not quite perfect. Bonner and Dodd [45], who counted the number of cells in small stalks and sori of *D. purpureum*, *D. mucoroides*, and *Polysphondylium pallidum* found that the relative size of the stalk is somewhat increased in the smallest fruiting bodies. This can also be seen in *D. discoideum* when a log-log plot is made of the volumes of stalks and corresponding spore masses over a wide size range (Fig. 3). The points in this figure can be fitted reasonably by a straight line, but the slope of the line is greater than 1; this is the so-called *allometric* relationship [46]. A slope of 1 would indicate constant proportionality; the observed slope indicates that the stalk is relatively larger in smaller fruiting bodies. Stenhouse and Williams [47] have also observed this phenomenon, although they disagree with previous investigators in claiming an additional, much more gradual increase in the stalk-spore proportion as the size of the fruiting body increases.

If the volumes of the *prestalk* and *prespore* zones in different-sized slugs are compared, they show a relation similar to that in the fruiting body (Fig. 3). This is especially dramatic in *D. mucoroides* whose slugs can be

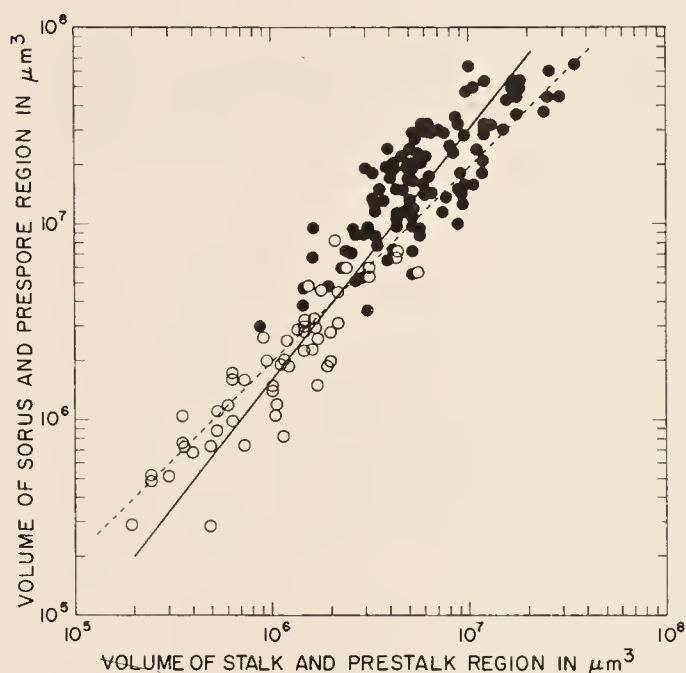


Fig. 3. The allometric relation between the volumes of prestalk and prespore tissue in migrating slugs (filled circles) and between the volumes of mature stalks and sori in fruiting bodies (open circles) in *D. discoideum*. The points are reasonably well-fitted by a single straight line (—) but noticeably less well by a line with a slope of 1.0 (---). This shows that prestalk zone and stalk are relatively larger in small slugs and fruiting bodies

measured over a range of volumes of almost three orders of magnitude [33].

In the same study [33], evidence was presented to suggest that the slug is partitioned into prestalk and prespore zones on the basis of volume rather than linear distance. If the *volumes* of the presumptive regions in *D. mucoroides* are plotted against each other on a log-log graph, one obtains a single line. If, on the other hand, the *lengths* of the prestalk and prespore zones are plotted, then the points from short, rotund slugs fall on a different line than those from long, cylindrical slugs. Thus it appears that it is the volume ratio, and not the length ratio, of prestalk and prespore zones which is held approximately constant in slime mold slugs.

There is one further bit of evidence that suggests that the significant feature of stalk-sorus proportioning is the volume relationship. In Fig. 3, both the prestalk-prespore relationship and the stalk-spore relationship are plotted on a single graph. Note that the two curves are confluent. This is quite remarkable when one remembers that spores are considerably smaller than prespores and that stalk cells are almost twice as large as prestalk cells [27, and unpublished data of JTB]. If all the presumptive cells turned into differentiated cells of the same type, the points for the mature stalks and sori would be on a line parallel, but considerably to the right of the

closed circles in Fig. 3. One can only conclude that some prestalk cells turn into spores during culmination, and this last-minute 'regulation' keeps the *volume* ratio of the differentiated cells identical to that of the presumptive ones.

#### *Factors Which Shift the Prespore : Prestalk Ratio*

Many treatments have been found to affect the ratio of stalk cells to spores formed in the cellular slime mold fruiting body, and a number of these cause corresponding changes in the prestalk-prespore pattern. These factors include temperature [35, 48], nutritional state [49, 50], and the concentrations of lithium and fluoride ions [32, 51, 52].

There are still cases in which the stalk : spore ratio is modified but the effects on the prestalk : prespore ratio are not known. Arginine [53] and ethionine [54] are known to affect the spore-stalk ratio, and mutants are known which show an excess of stalk cells [55] or spores [56, 57]. Studies are needed of the prespore-prestalk pattern under these conditions.

There are also some curious observations from experiments in which a fruiting body is constrained in some way. For instance, Shaffer [58] found that if the rising fruiting bodies of *Polysphondylium violaceum* got caught in an oil-water interface they differentiated entirely into stalk cells. Farnsworth reports that cells trapped in a small cellulose tube also turned into stalk cells while a plastic bar pushed two-thirds of the way into a rising cell mass occasionally gave 100% spores [59].

#### *Development of a Prestalk-Prespore Pattern Without Normal Morphogenesis*

When starving *Dictyostelium discoideum* cells are shaken slowly in dilute buffer they form clumps of up to  $10^5$  cells, by-passing normal aggregation. Gerisch showed that these balls of cells become polar if they are brought to an air-water interface, the polarity being determined by the interface itself [60]. It had been previously shown that such clumps of cells become polarized even if care is taken to see that they remain submerged [61]. It has now been found [37, 39, 40] that under certain conditions, including high oxygen tension, the differentiation of mature stalk cells and spores occurs under water. After a number of hours in a roller tube the cell mass, an irregular spheroid, elongates slightly, and a slug-shaped region becomes visible inside a layer of peripheral cells.

The slug-shaped region has prestalk and prespore zones with roughly the normal positions and proportions. The peripheral cells differentiate into mature stalk cells as do the prestalk cells of the slug-shaped region; this differentiation progresses from the outside of the cell mass inwards. As stalk differentiation proceeds, the prespore zone loses its coherence; the spores ultimately appear in irregular groups among the mature stalk cells. A core of undifferentiated cells with prestalk and prespore zones in approximately normal proportions may persist throughout the period of 'development'. These results argue against the idea that prespore-prestalk pattern formation depends in any way on aggregation or slug migration. The fact that the final structure produced contains an irregular distribution of mature spores and stalk cells suggests, however, that the formation of a normal fruiting-body requires the usual sequence of culmination stages.

#### **Positionally Varying Properties other than the Prestalk-Prespore Pattern**

##### *The Slug Tip*

The cellular slime mold slug has a nipple-shaped structure at the anterior end which is called the *tip* or *conus*. The tip is formed before the slug proper and persists until near the end of culmination. The tip has been considered to 'organize' the slug, as shown in experiments in which extra tips are implanted in a slug: In such cases the slug splits in as many fragments as tips are present, and each tip leads one of the fragments away [30, 62]. The slug tip appears to produce acrasin [63], that is, 3',5'-cyclic AMP [62, 64, 65]; its organizing ability is sometimes thought to reflect chemotaxis of slug cells towards this [62, 65–67, but see 68].

It is difficult to be certain of the boundaries of the tip region in any given slug, but it is nonetheless clear that small slugs have smaller tips than large slugs, so that the proportions of the tip-body pattern are at least roughly independent of the slug size. If a tip is removed from a slug, a new tip will be regenerated. The tip-body pattern thus has at least two of the features of the prestalk-prespore pattern: proportion invariance and the ability to regulate. It is attractive to suppose that the two patterns are related, and that the prestalk-prespore pattern may be approached experimentally by studying the formation of the tip.

Tip formation has been studied by Farnsworth, who has shown that the tip inhibits the formation of a second tip in the same cell mass [69]. Durston has further found



that the intensity of this inhibition and the resistance of slug tissue to it are graded properties in the slug: both inhibition and resistance to inhibition are highest in the front of the slug and decline towards the rear [70]. In these respects tip formation in cellular slime mold slugs is similar to head and foot formation in *Hydra* [71–76], and models which have been developed to account for head and foot formation in *Hydra* may be applicable as well to tip formation in cellular slime mold slugs. Two of these models, the *followup-servo model* [74] and the *activator-inhibitor model* [77] are attractive models for both tip formation and prestalk-prespore patterning; these will be discussed below (see Models).

It is important to bear in mind that the exact relationship between the tip-body pattern in cellular slime mold slugs and the prestalk-prespore pattern is very much an open question. It is possible, for instance, that one of these patterns is 'primary', and leads directly to the formation of the other; the tip may 'organize' the prestalk zone (as envisioned by some 'positional-information' models; see below), or, alternatively, the tip may be formed by special morphogenetic activities of prestalk cells [37]. It is also possible that the two patterns influence one another without a clear cause-and-effect relationship, or that the patterns are entirely independent. Further research is needed to resolve this issue.

### *Cyclic AMP and Pattern in the Slug*

Various lines of evidence suggest a nonuniform distribution of 3',5'-cyclic AMP in the cellular slime mold slug. In some early experiments [63] slugs were cut in two or three pieces and put in the presence of aggregation-competent amoebae; these were attracted towards the pieces. Measurements of this attraction made *within a few minutes* of cutting suggested that the slug contains considerably more chemotactic attractant, presumably cyclic AMP, in the front than in the rear. Rubin and Robertson [62] have recently confirmed this result in an experiment in which only tips and slug middles were used; the tips attracted amoebae *immediately*, while the middles did not. Periodic attraction of the amoebae, suggestive of relaying, was observed at later times in both cases. It appears that newly-formed slugs give a different result: Each portion secretes the same amount of cyclic AMP [48, 78].

Using another approach, indirect immunofluorescent staining, Pan and coworkers [79] have observed positional variation in the concentration of bound cyclic AMP in *Dictyostelium* slugs. A gradient-like distribution of fluorescence was found in young slugs, with higher

levels in the front. In older slugs, fluorescent staining revealed two regions which appeared to be identical to the prestalk and prespore zones. Most recently, Brenner [80] has confirmed previous work of Garrod and Malkinson [81] showing that the total concentration of cyclic AMP (bound and unbound) is about 40% higher in the front third of the slug than in the rear two-thirds. There were indications that the pattern might be step-like, as if correlated with prestalk-prespore zonation.

These findings consistently support the idea that cyclic AMP levels are position-dependent in cellular slime mold slugs. It is necessary to be cautious, however, in assigning a morphogenetic role to cyclic AMP on the basis of this evidence, since it is not clear whether the cyclic AMP pattern is a cause or a consequence of prestalk-prespore differentiation. It is known that cyclic AMP can cause free amoebae to become stalk cells [82–85], but this 'induction' may not be direct, since it appears to require a dialyzable factor produced by the amoebae as well as the cyclic AMP [85]. The view that cyclic AMP is a prestalk cell 'morphogen' is also beclouded by the findings that many of the cells which ultimately become stalk in induction experiments in fact initially differentiate as prespores [86], and that both stalk cells and spores are formed when Sephadex beads impregnated with cyclic AMP are implanted in migrating slugs [87].

### *Cell Polarity and Cell Contact*

Cellular slime mold cells are aligned in a polar fashion during aggregation. In aggregation streams observed under phase contrast it is easy to see that each cell has its contractile vacuole behind the nucleus: An arrow drawn from the contractile vacuole to the nucleus always points in the direction of movement. Shaffer showed that aggregating cells in fact make end-to-end attachments [88], and Beug, Gerisch, and coworkers [89, 90] have demonstrated specific adhesive sites which appear to be responsible for these. The structure of the outer membrane is different at the two ends of amoebae in aggregation streams, and these cells show 60-angstrom filaments in the anterior ends only [91]. It is not known to what extent cell polarity persists in the slug.

Some early experiments suggest that the mass cell movements of late aggregation are controlled by direct cell contact: If a portion of an aggregation stream is cut out, reversed 180°, and put back, it disintegrates unless it is stuck onto the amputated central stream. When it is attached, the cells appears to be 'pulled' into the center [61].



### *Positional Variation Due to Sorting-Out*

It has long been recognized in animal developmental systems that the fate of a cell is a function of its position in some cases, while in others, the final position of a cell is determined by its differentiated properties. In the latter instance, there is a sorting out of wholly or partially differentiated cells by cell movement. There is mounting evidence that cell sorting occurs during slime mold development and that it plays a role in slime mold pattern formation. Bonner first demonstrated sorting in the form of a nonrandom distribution of mutant cells within *Dictyostelium* slugs [92]. Takeuchi mixed cells from the front of thymidine-labeled slugs with unlabeled cells from the rear of other slugs and allowed aggregation to occur. The label in the resulting slugs had a gradient-like distribution, with the highest labeling in the front, indicating that the majority of the originally anterior cells had come to rest in the front of the new slugs [93].

Further experiments of Maeda and Maeda show that starving preaggregation cells can be separated by centrifugation into front-preferring and rear-preferring types [94]. This suggests that positional sorting is not due to a 'positional memory' which is 'set' the first time a cell resides in a slug, but to differences between front- and rear-preferring cells which are already present among preaggregation amoebae. Sorting propensities seem to be extremely stable: when front-preferring cells and rear-preferring cells are forced to culminate separately, the cells that germinate from the resulting spores retain the original sorting-out preferences [93]. The differences are erased only if the amoebae are allowed to undergo vegetative growth.

Since sorting-out clearly occurs in cellular slime mold development and since the initial formation of the prestalk-prespore pattern has been interpreted as sorting-out, it is tempting to suppose that prestalk-prespore patterning is in fact due to sorting. One might thus hypothesize that 'prestalk and prespore amoebae' are already distinct from one another before aggregation, and that during slug formation these preferentially take up positions in the front and rear of the slug, respectively, constructing the prestalk-prespore pattern. A disadvantage of this sort of model is that it is of very little help in explaining the phenomena of regulation, which seem to involve conversion of prestalk cells to prespores, or vice versa. The idea that pattern formation and pattern regulation proceed by entirely different mechanisms does not seem particularly attractive.

Extensive studies of sorting by Leach and coworkers [95] in fact suggest that sorting alone cannot account for the formation of the prestalk-prespore pattern. These

workers investigated the influence of nutritional state on sorting behavior. Four nutritional conditions were studied, and these could be ranked in order of front-preference, so that in any pair, the member which was higher on the list would sort out to the front of a composite slug. A simple interpretation of these results is that cells sort according to the value of a *continuously variable-parameter*, related to nutritional state, and not simply according to established prestalk or prespore identities. The value of this parameter in a particular cell *in relation to its value in the other cells of the slug* appears to determine the cell's fate.

This strongly suggests that a mechanism exists *in addition to the sorting mechanism* which can, in effect, examine the spectrum of cells in an aggregate and assign individual cells to either prestalk or prespore pathways, depending on the overall composition of the cell mass. It is easy to imagine that a mechanism of this sort could also bring about regulation of the prestalk-prespore pattern without the need for any further sorting.

### **Models**

The prestalk-prespore pattern in cellular slime mold slugs can be accounted for in at least three quite different ways:

1. In a *cell-contact model*, put forth by McMahon, interactions between adjacent cells give rise to patterning in the slug as a whole [96].

2. *Positional information* models assume that a continuous gradient of some sort is formed along the length of the slug. Cells then judge their positions from the local gradient level and develop prestalk or prespore tendencies accordingly. Such models have been advanced by Ashworth [97] and by Loomis [98]. In addition, Durston and Vork [99] have advocated applying Wolpert's [74] 'followup-servo' model, a positional-information model developed for *Hydra*, to cellular slime mold slugs.

3. *Reaction-diffusion models* generate pattern using systems of diffusing substances; the concentrations of these substances feed back on their production rates. A model of this sort developed by Gierer and Meinhardt [77] can be applied to cellular slime mold slugs as a mechanism for regulating the proportion of prestalk to prespore tissue. We will discuss this model as the *activator-inhibitor model* here.

It is interesting to note that these various kinds of models have been used to account for pattern formation in many different developing systems [100–106]. They are in fact three very general ways of looking at the

problem of pattern formation. Cellular slime molds thus present the general problem of biologic pattern formation in microcosm.

The various models to be discussed here differ somewhat in their ability to account for the phenomena of prestalk-prespore patterning that we have presented above; we will attempt to point these differences out as we discuss the models. The activator-inhibitor model appears at present to be fully satisfactory, while none of the others are, but this situation could change as new information becomes available.

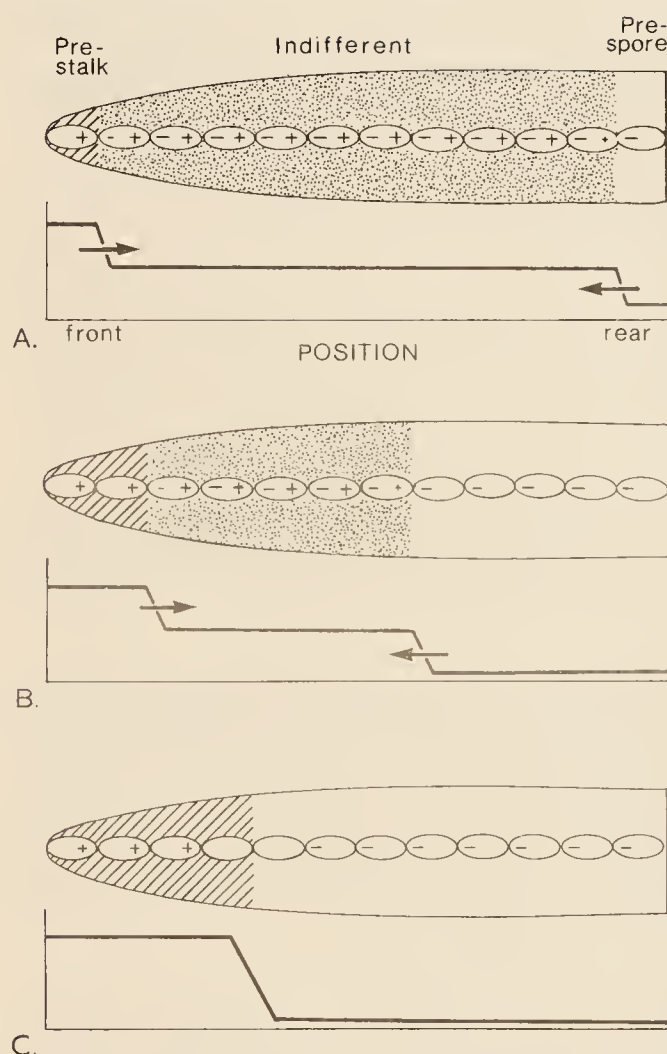
The models are so widely divergent in mechanism that it should be possible to distinguish among them with relatively simple experiments. Two approaches in particular seem likely to be fruitful: experiments involving transplantation of tissue from one slug to another, and the isolation and study of prestalk-prespore pattern mutants. Transplantation experiments are feasible with cellular slime molds [62, 70, 99]. The isolation of mutants is also feasible: Available methods of mutagenesis give per locus mutation rates on the order of  $10^{-3}$  with high viability [51]; such high rates make it possible to search for particular mutants by simple screening even in cases where no selection can be devised. We will discuss the transplantation phenomena and classes of mutants which each of the models predicts along with the models themselves.

A point we would like to make before proceeding is that the proposal that pattern formation involves cyclic AMP (as many have suggested) is compatible with all of the models that we will discuss here; none of the models is specifically a 'cyclic AMP model'.

### The Cell-Contact Model

**General Scheme.** McMahon [96] has proposed that cells of the slime mold slug interact with their immediate neighbors to the front and to the rear in such a way as to generate an overall pattern in the concentration of an intracellular morphogen called 'A'. The interactions are assumed to be mediated by a system of cell surface molecules (which it is suggested might include membrane-bound adenyl cyclases and phosphodiesterases). The proposed interactions are quite complex, but appear to reduce to the following:

1. Each cell influences the cell to the rear to *decrease* its concentration of A.
2. Each cell influences the cell in front to *increase* its concentration of A.
3. The strengths of the two influences are controlled by the level of A: when this level is high, the rearward



**Fig. 4.** Formation of the prestalk-prespore pattern according to the cell-contact model. A morphogen is assumed to be present within the cells, and its level is assumed affected by neighboring cells. The neighbors' effects are schematized by + and - symbols within the cells; these effects initially balance in cells within the slug. (Top) Pattern formation is initiated by an imbalance of neighbor-effects in the cells at the two ends. (Middle) Changes in the morphogen level cause cells on the ends to 'uncouple' from their neighbors; this unbalances cells within the slug. (Bottom) A stable state is reached when all of the cells in the slug are unbalanced. For further discussion, see the text

(negative) influence disappears, while the forward (positive) influence disappears at low A concentrations. The influences balance at a moderate level of A and this level is stable at any position away from the ends of the slug.

Pattern formation occurs in the model on account of these assumptions and on account of the special situations of cells at the front and the rear (Fig. 4). A cell at the front, with no cells in front of it, receives no negative influence. As a result, its A level will increase. The front cell then ceases to have a negative influence on the cell to its rear, and the level of A in the second cell thus increases. Repetitions of this cycle cause a wave of in-



creasing  $A$  concentration (a 'positive wave') to sweep from the front of the slug rearwards. At the same time, a 'negative wave' of decreasing  $A$  concentration sweeps in from the rear. These two waves meet in the middle of the slug, and there is thereafter a region of high  $A$  concentration in the front and a region of low  $A$  concentration in the rear. Since the front zone has no influence to the rear, and the rear zone has no forward influence, the boundary separating the regions of high and low  $A$  concentration is stable. The ratio of the sizes of the two regions is determined only by the relative rates of propagation of positive and negative waves, and is thus independent of the overall size of the slug. McMahon suggests that  $A$  controls prestalk-prespore differentiation directly, with the result that a prestalk zone is formed in the front of the slug while a prespore zone is formed in the rear.

McMahon has suggested that cyclic AMP could play the role of  $A$  in cellular slime mold slugs. We feel that it is important to recognize, however, that the essential features of the model, namely the cell interactions which bring about pattern formation, do not necessarily follow from what is now known about cyclic AMP metabolism. The model is thus best regarded as a cell-contact model; this would appear to be consistent with McMahon's expressed views.

*Problems.* Several troublesome points can be raised about the cell-contact model. The first is that it seems unlikely that the model can account for pattern regeneration or proportion regulation in cellular slime mold slugs. This problem arises because the prestalk-prespore boundary formed in the model is stable, and its stability follows from local interactions which do not depend on any overall properties of the slug. There is therefore no way in which a disturbance in the overall pattern (such as removal of a part of the prestalk or the prespore zone) could bring about repositioning of the prestalk-prespore boundary. This problem seems to be a direct consequence of the assumption, basic to cell-contact models, that patterning is controlled by local cell interactions only.

It is clear that one could get around this problem by assuming that the entire pattern formation process is reinitiated spontaneously at short intervals; regulation would then proceed without any need to detect disturbances in pattern. Models incorporating such an assumption, however, have problems of their own. If one assumes that patterning cycles occur quite frequently — every few minutes or less — one has to envision the visible prestalk-prespore pattern as a sort of a long-term average of the continually decaying and reappearing

pattern in the concentration of  $A$ . During regulation, one would expect that the pattern in  $A$  would readjust rapidly and that the visible prestalk-prespore boundary would then 'fade out' at the old position while simultaneously 'fading in' at the new. This does not seem consistent with the observation that the boundary moves continuously from the old position to the new one during regulation (see above). If one envisions instead that repatterning occurs at intervals of an hour or so, then one is free to assume that the positive and negative waves 'switch' any cells they encounter in an inappropriate predifferentiation state; it is then possible to account for the appearance of a moving prestalk-prespore boundary. In this case, however, one would expect to observe a variable-length delay before boundary movement began, corresponding to the waiting time before a new cycle of patterning. The maximum length of this delay should be as great or greater than the time involved in the visible processes of regulation, and would therefore be readily observable. Unpublished observations (HKM) do not support the existence of such a delay, although these studies may not be detailed enough to rule it out definitively.

A second way to get around the regulation problem in cell-contact models would be to assume cell interactions complex enough to mimic gradients, negative feedback, or other global phenomena in the slug. Cell-contact models modified in this way are probably best considered as *hybrid models* (see below).

Two further problems with the specific cell-contact model proposed by McMahon concern its ability to account for the prestalk-prespore pattern quantitatively. The model would divide up the cellular slime mold slug into prestalk and prespore regions on the basis of length and not volume; this is in conflict with the finding (see above) that the ratio of prestalk to prespore tissue volumes is closer to constant in slime mold slugs than the length ratio. The model as proposed would also fail to account for the allometry of the prestalk-prespore ratio (i.e., its dependence on the overall size of the slug). It is not clear to us that other cell-contact models could circumvent these difficulties.

*Characteristic Phenomena.* Two features of the McMahon cell-contact model could give rise to phenomena which, if observable, would strongly support it. The first of these features is the model's sensitivity to misbehavior on the part of even a small number of cells. For instance, *chain terminator mutants* [96] which block the propagation of positive or negative waves, and *false initiator mutants*, which initiate such waves at sites other than the ends of the slug, should grossly disturb pattern

formation even if they are diluted with a large excess of wild type amoebae. It seems to us that sensitivity to a few mutant cells is likely to be a general feature of cell-contact models, since in such models single cells can act as the sole or major source of developmental information for many other cells. We know no reason to expect similar sensitivity from positional information or activator-inhibitor models.

The McMahon model also makes distinctive predictions in the case in which a prestalk-prespore boundary is facing in the 'wrong' direction (with prespore cells in front and prestalk cells to the rear). Cells would be in full communication across such a reversed boundary, with the prestalk cells attempting to elevate the concentration of *A* in the front zone while the prespore cells attempted to bring the level of *A* in the rear zone down. If these influences failed to cancel exactly, the net effect would be movement of the boundary in one direction or the other. This movement should be easy to demonstrate. If, for example, reversed boundaries propagate into prespore tissue and convert it to prestalk, then placing a small transplant of prestalk tissue in the prespore zone should lead to the formation of a stripe of prestalk tissue extending from the transplant forward to the main prestalk-prespore boundary. If reversed boundaries migrate into the prestalk tissue, on the other hand, prespore transplants to the prestalk zone should induce prespore stripes.

Since cell-contact models could presumably be constructed in which a reversed prestalk-prespore boundary was stable, the production of stripes following transplantation is not necessarily expected of models of this class. As far as we can see, however, such stripes could not be explained by the positional information or activator-inhibitor models; the observation of stripe formation following transplantation would thus strongly support cell-contact models. It would of course be important that any claim that stripes had been demonstrated experimentally be supported by evidence that the stripes were formed by prestalk-prespore conversion and not by migration of the transplanted cells.

### Positional-Information Models

**Basic Scheme.** In *positional-information models* it is assumed that pattern formation is a two-step process. In the first step, a concentration gradient of a substance (or a gradient in some other parameter) is formed along the length of a tissue which is to undergo patterning. The level of this gradient in any cell in principle informs that cell of its position in the tissue. In the second step, called

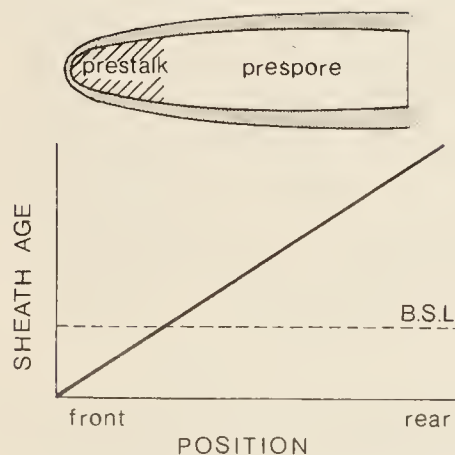


Fig. 5. Prestalk-prespore patterning according to the surface-sheath model. A gradient is present in the age of the surface sheath; a boundary-specifying level (B.S.L.) of this gradient divides the slug into prestalk and prespore zones

'gradient interpretation', cells acquire commitments and/or differentiate according to their positions. The levels of the gradient at the ends of the tissue are usually assumed to be independent of the tissue size, so that all of the gradient levels present in a long piece of tissue are also present in a short one, and all zones in the pattern are automatically proportioned to the size of the tissue as a whole.

Here we will discuss two models which employ the concept of positional information to explain patterning in the cellular slime mold slug, the *surface sheath model* and the *stable/labile-gradient model*. Positional information is generated in rather different ways in the two models, which therefore differ in their predictions.

**The Surface Sheath Model** (Fig. 5). The cellular slime mold slug encloses itself in a semi-rigid, acellular matrix called the *slime sheath* or *surface sheath*. The sheath is stationary during slug migration: the slug manufactures sheath at its tip and crawls through, the empty sheath remaining after the slug has passed. Since the sheath is manufactured at the slug tip, the sheath in this position is always composed of new material; the sheath becomes progressively older toward the posterior end of the slug.

Loomis has proposed that this age gradation in the surface sheath provides positional information to the slug cells in an indirect fashion: A diffusible molecule is thought to escape from the slug through the sheath, the permeability of which is thought to decrease with age; the concentration of the substance remaining in the slug gives a local indicator of the sheath age and therefore of anterior-posterior position. In a secondary gradient reading process, cells which experience a concentration of the diffusible substance which is less than some



threshold value become prestalk cells; other cells become prespores [98]. Sussman and Schindler have proposed a similar model, in which ammonia produced in the slug escapes through the thin sheath of the tip. The absence of ammonia in the tip region is thought to stimulate the production of cyclic AMP, which in turn controls cell differentiation [65]. An ingenious extension of these assumptions allows the authors to explain, in a qualitative fashion at least, the major events of culmination as well.

Ashworth has proposed a related theory in which the sheath undergoes progressive chemical modification as the slug passes through; here the positional information is provided in an unspecified way by the local degree of sheath modification [97].

In both the Loomis and the Ashworth model, constancy of the prestalk-prespore proportion results from the fact that small slugs migrate more slowly than large slugs, so that the gradient (of sheath age or chemical modification) is steeper in small slugs. Readjustment of proportion in the front half of a bisected slug would then be a consequence of a decrease in the migration rate; pattern regeneration in the rear would follow upon the formation of a new slug tip, and thus young sheath, at the front end of the piece. Too little is known about sheath biology to say whether this mechanism would divide a slug into zones on the basis of length or volume, or whether the proportioning would be sensitive to the total size of the slug.

An earlier formulation of the Loomis model, which relied on a gradient in surface sheath thickness to provide positional information within the slug, has been shown untenable by Farnsworth and Loomis [107] who demonstrated that the slope of the thickness gradient is the same in large and small slugs.

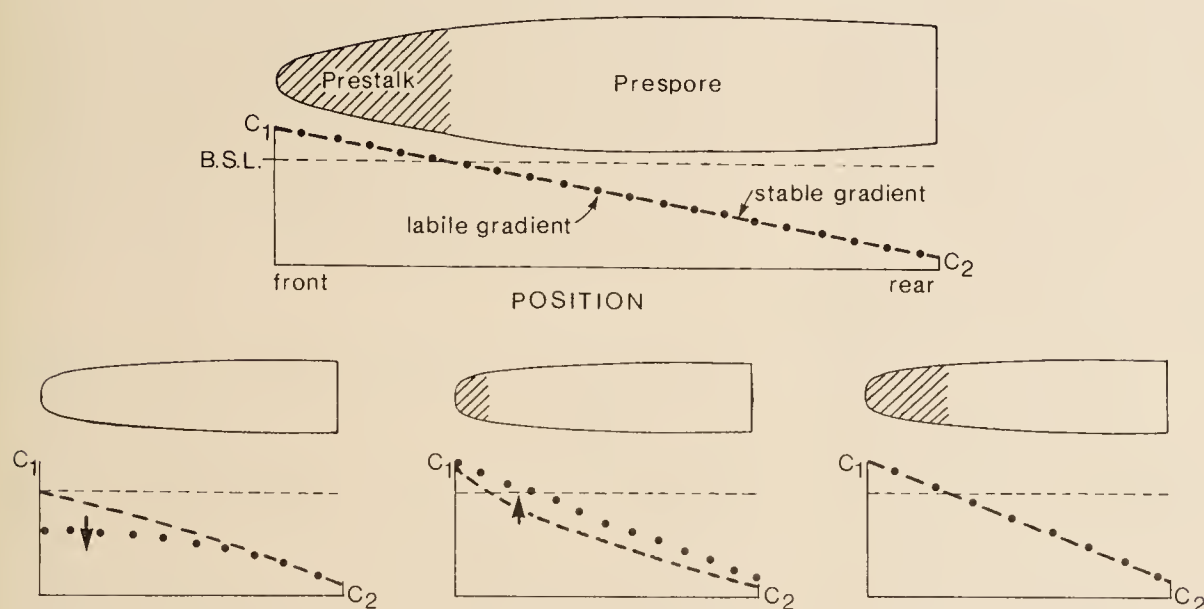
**Problems.** A problem with surface-sheath models is that they suggest that migration should be essential for pattern regeneration and proportion regulation; this appears to contradict published data. Bonner and coworkers have depicted pattern regeneration (demonstrated by PAS staining) in temporarily stationary cell masses [1]. Experiments with submerged-culture aggregates (see above) show that a prespore-prestalk pattern can be formed in the complete absence of migration. Florescent antibody work suggests that the prestalk-prespore pattern is normally formed before migration begins [57]. The fact that normal fruiting bodies are produced even if the slug stage is bypassed entirely [108] clearly shows that migration is not essential for the orderly division of the cell mass into differentiated stalk cells and spores.

Surface-sheath models also have problems explaining the results of Sampson [36, discussed above] who showed that disaggregated fragments of a slug, allowed to culminate, gave the same proportions of stalk and spore cells as fragments with the surface sheath left intact. An anterior fragment, in particular, gave a disproportionately large stalk whether or not the surface sheath had been destroyed. This result strongly suggests that there are factors other than the surface sheath which are active in controlling the stalk-spore proportion in cellular slime molds.

*The Stable/Labile-Gradient Model* (Fig. 6). A particularly interesting alternative model employing the positional information concept was first proposed by Wolpert as the 'followup-servo model' [74] to account for regeneration in *Hydra*. In this model positional information is defined between two *boundaries*, which in cellular slime molds would be specialized regions at the two ends of the slug. These two boundaries act as a source and a sink (respectively) for a diffusible morphogen, so that a linear concentration gradient in morphogen concentration is formed between them [109]; the concentration of this morphogen is assumed to provide positional information to the slug cells.

Two further assumptions are made to give the model the capacity to regenerate its boundaries. One is that a second gradient is present in the slug, the level of which is usually a long-term average of the level of the first. Due to time averaging, the second gradient (the 'stable gradient') 'follows' small adjustments in the shape of the first ('labile') gradient. An additional assumption is that if a large difference arises between the stable and labile gradient values in any position, the stable gradient value in that position *diverges*: It changes in the direction opposite that which would be dictated by follow-up behavior. Thus, if the morphogen source is removed, and the concentration of the labile morphogen in the front of the slug drops well below the stable gradient value, the *stable* gradient value in the frontmost cells begins to *increase*. This increase is assumed to continue until these cells attain the stable gradient value characteristic of the front boundary. The frontmost cells then become boundary cells, producing the diffusible morphogen. The labile gradient is thus re-established and follow-up behavior resumes throughout the system. Regeneration of the morphogen sink results in a similar fashion from an increase in the diffusible morphogen concentration following its removal.

To the best of our knowledge, no mechanism has been proposed to account for the initial establishment of the source and sink.



**Fig. 6.** Regulation of the prestalk-prespore pattern after the prestalk zone is removed, according to the stable/labile gradient model. (*Above*) In the normal state, the labile gradient extends from a source (with morphogen concentration  $C_1$ ) to a sink (with morphogen concentration  $C_2$ ). The stable gradient runs with the labile gradient, and a boundary-specifying level (B.S.L.) of the stable gradient defines prestalk and prespore zones. (*Below left*) After removal of the prestalk zone, including the morphogen source, the labile gradient value drops in the anterior part of the slug. (*Below, center*) In a region in which the labile gradient value has dropped sufficiently below the stable value, the stable gradient value increases; as a result, prestalk tissue reappears. The morphogen source is regenerated and labile gradient at the anterior end rises again to the level  $C_1$ ; a linear labile gradient is reestablished. (*Below right*) The stable gradient value follows the labile value, and the prestalk-prespore boundary moves to the correct position

**Problems.** Two specific drawbacks of the stable/labile-gradient model need to be considered. The first is that the model's use of a source-sink gradient leads to the prediction that the ratio of prestalk to prespore volumes will depend systematically on slug shape, a prediction that conflicts with observations discussed above. This prediction follows from the fact that in a source-sink gradient the flux of molecules diffusing across all planes that pass between the source and sink must be the same. In a slug of irregular shape, this means that the slope of the gradient is steeper where the cross-sectional area is less. Since the gradient is predicted to be steepest in the thinnest parts of the slug, these parts influence the position of the prestalk-prespore boundary more than thicker parts do. The gradient would have to be *shallower* in thinner parts of the slug, rather than *steeper*, to make the ratio of prestalk and prespore volumes independent of slug shape, as has been observed (see above).

A second problem is that a source-sink gradient cannot account for the allometry of the prestalk-prespore pattern in a simple fashion; if anything, one would expect an effect in the direction opposite to the one observed. The demands on a source and sink would be greatest when the slug was small, since the morphogen gradient would be steepest — and the flux of diffusing molecules therefore greatest — in a small slug. At the same time, the tissue available for the source and sink in

a small slug is least. If the source and sink in small slugs were to fail to keep up with the demands on them, the morphogen gradient would flatten, and this would cause a decrease in the proportion of the slug which was prestalk. This behavior does not agree with the observation that the prestalk zone in very small slugs is proportionately enlarged.

**Transplantation Properties.** According to the stable/labile gradient model, it should be possible to cause a slug to form extra boundaries simply by transplanting tissue from one position to another. For example, if tissue were moved from near the source to near the sink, the labile gradient in the transplant would soon assume a value substantially below the stable gradient value, and the transplant would then be expected to form a new morphogen source. Sinks should be formed in a parallel fashion if tissue is moved in the opposite direction. One of these boundaries should appear to 'induce' prestalk character, and the other prespore character, in the surrounding tissue. Durston's demonstration of gradients of inhibition of tip formation and of resistance to this inhibition [70] (see *The Slug Tip*) are strongly reminiscent of these predictions, and suggest that the tip be interpreted as the front boundary of the slug. To the best of our knowledge there is no experimental evidence that directly supports the existence of a 'rear boundary'.



*Mutants and Positional-Information Models.* Positional-information models in general predict that distinctive phenomena might be observed in experiments involving mixing wild-type cells with mutants which display altered prestalk-prespore ratios. Prestalk-prespore ratio mutants, according to positional information models, could in principle have abnormalities in the generation of positional information (generation of a gradient of incorrect level or slope) or in its interpretation (using an abnormal gradient level to specify the prestalk-prespore boundary). If cells of a *gradient generation mutant* were mixed with wild-type cells, a compromise gradient would result, and slugs would show a prestalk-prespore boundary located between the normal and mutant positions. If cells of a *gradient reading mutant* were mixed with wild-type cells, however, the mutant and wild-type cells would read the gradient differently and the resulting slugs would show *two* prestalk-prespore boundaries, one at the normal and one at the mutant position. Compromise boundaries could be accounted for by other models, but the double-boundary phenomenon could not, as far as we know, be explained by anything but a positional information model. The isolation of even one gradient-reading mutant would thus strongly support the positional information hypothesis.

#### *The Activator-Inhibitor Model*

Gierer and Meinhardt [77; see 105 for review] have proposed a general model in which patterns are created and stabilized by diffusing substances. A specific formulation of this general model is capable of proportion homeostasis. This specific model, applied to the cellular slime mold slug, can account for the formation and regulation of the prestalk-prespore pattern, and can further account for its quantitative aspects — the negative allometry of the prestalk zone and the fact that the prespore-prestalk ratio is most nearly constant when expressed in terms of volumes. The model also provides an interpretation for the role of sorting-out in cellular slime mold pattern formation. Because this model has not been presented previously as a cellular slime mold model we will discuss it here at some length.

We emphasize that the formulation discussed here is not the only formulation of the Gierer-Meinhardt model; there are other versions which do not show proportion homeostasis, but which may be used to provide positional information in systems in which proportion regulation does not occur [104].

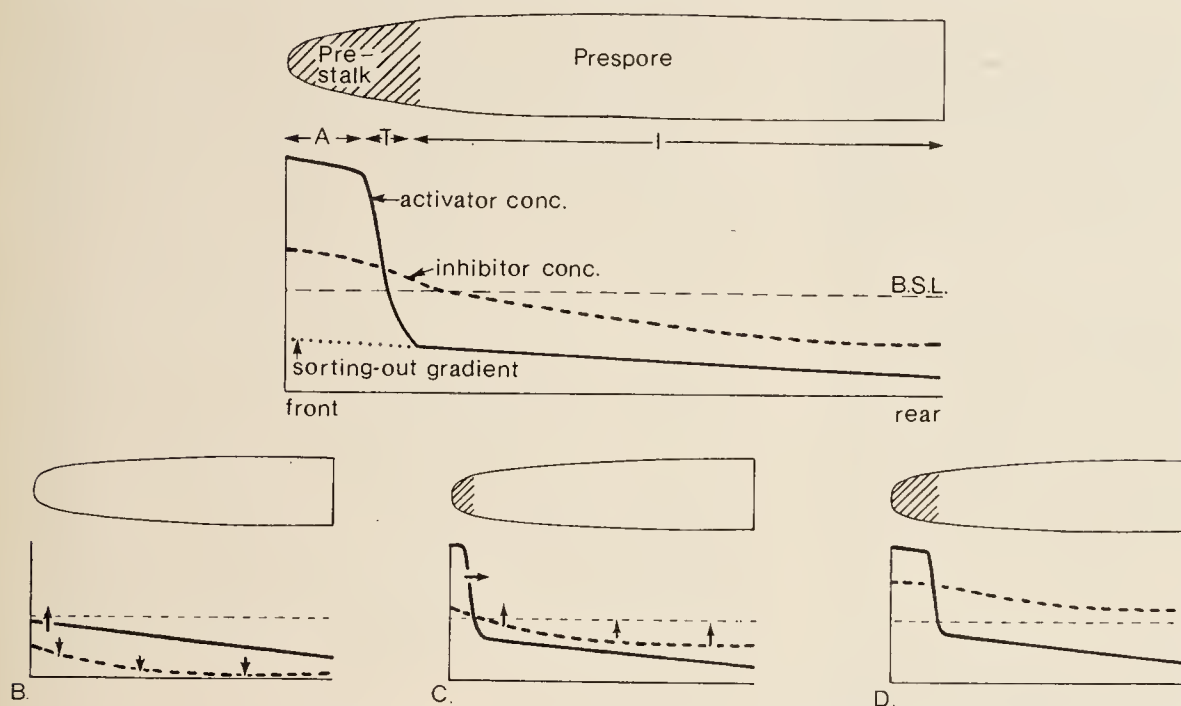
The Gierer-Meinhardt model, unlike many other biologic models, is designed *more for mechanistic simplicity*

*than for conceptual simplicity.* The definitive form of the model is a set of differential equations, the behavior of which is best determined by computer simulation. For the sake of stylistic consistency we will present the model in intuitive terms here. We urge the reader to bear in mind, however, that this method of presentation necessarily does not give full justice to the model's simplicity and precision.

*The Basic Scheme.* The model that we will consider (Fig. 7) employs two diffusing substances, an *activator* and an *inhibitor*. The activator is autocatalytic: It stimulates its own production. The activator also indirectly inhibits its production through a feedback loop involving the inhibitor: The activator stimulates inhibitor production, and the inhibitor in turn competitively blocks the activator autocatalysis. Both the activator and the inhibitor are assumed to be broken down continuously. Autocatalysis, inhibition, and breakdown proceed according to a specific kinetic scheme which is consistent with simple enzymatic mechanisms.

The activator and inhibitor are assumed to have different diffusion ranges; this is due to differences in the ratio of their diffusion and breakdown rates. The inhibitor has a potential diffusion range which is larger than the slug, so that its concentration, to a first approximation, does not vary with position. The activator, however, diffuses only a few cell diameters before being broken down. Since most of the inhibitor produced by any small group of cells diffuses away, while the activator that these cells produce remains, the activator-inhibitor system on a local scale is primarily autocatalytic. The activator concentration thus displays a strong local positive feedback, and a small group of cells tends to be in either an 'activated' state, in which the activator concentration is high (limited only by saturation of the activator production process), or an 'inhibited' state, in which the activator concentration is near zero. In general, slugs contain both activated and inhibited tissue, and there are thus also narrow 'transition zones' of intermediate activator concentration which separate the two tissue types.

*Homeostasis of the Prestalk: Prespore Proportion.* Since the activator stimulates inhibitor production, inhibitor is produced primarily in activated tissue. The inhibitor is broken down throughout a slug, however, with the result that its concentration is a measure of the proportion of a slug's tissue that is activated. In a slug with a large fraction of activated tissue, the inhibitor level is high enough to destabilize activated tissue, causing some of this to become inhibited. In a slug with little activated tissue,



**Fig. 7.** Regulation of the prestalk-prespore pattern according to the activator-inhibitor model. (*Above*) In the undisturbed slug, the activator shows a three-part pattern: activated (A), transitional (T), and inhibited (I) zones. Sorting-out produces a gentle activator gradient which is superimposed on this pattern. The inhibitor is produced primarily in the activated zone but diffuses throughout the slug. A boundary-specifying level of the activator gradient separates the slug into prestalk and prespore zones. (*Below left*) The inhibitor concentration drops after removal of the prestalk zone. In response the activator concentration rises; this is especially fast at the anterior, where its concentration is initially highest. (*Below, center*) A new activated zone is created in the anterior of the slug. Inhibitor is produced in this zone, and the inhibitor level throughout the slug rises. The activated zone expands via rearward migration of its posterior boundary. Where the activator concentration exceeds the boundary-specifying level, prestalk tissue reappears. (*Below right*) The inhibitor has attained a level sufficient to stop the expansion of the activated zone. The activator pattern and the prestalk-prespore pattern are now stable

conversely, the inhibitor level is low enough so that the residual activator present in inhibited tissue becomes autocatalytic, and some inhibited tissue becomes activated. In either case these changes continue until the inhibitor concentration is at a 'critical level' at which the system is stable. There is thus a homeostatic regulation of the proportion of activated tissue in the slug. Conversion of activated tissue to the inhibited state or vice-versa within an existing pattern occurs preferentially at transition zones; thus regulation occurs via migration of the prestalk-prespore boundary, and the two-zoned configuration of the prestalk-prespore pattern, once established, would be preserved.

By adjusting the values of constants in the model, the proportion of tissue activated at the critical inhibition level may be made the same as the proportion of prestalk tissue in a cellular slime mold slug. Thus if one supposes that prestalk tissue is in the 'activated' state, while prespore tissue is 'inhibited', the activator-inhibitor system can account for the regulation of the prespore-prestalk proportion in cellular slime mold slugs.

*Control of Polarity and Pattern Configuration: The Sorting-Out Gradient.* Additional assumptions are nec-

essary to ensure that activated and inhibited tissue will have an appropriate *spatial arrangement*, that is, that there will be a single activated zone in the front of the slug and a single inhibited zone in the rear. We propose that spatial ordering is brought about *in an indirect fashion* by sorting-out. Specifically, we envision that individual slime mold amoebae produce activator in a low-level secondary process which is unaffected by the activator-inhibitor interaction; this process is analogous to the 'basic activator production' envisioned in the Gierer-Meinhardt model for *Hydra* [77]. We envision that the rate of basic activator production in slime mold amoebae is variable, and further that it is correlated with the parameter which controls cell sorting, so that cells with higher basic production tend to sort to the front of a slug. The direct result of cell sorting would then be a gradient in basic activator production and a corresponding low-level gradient in the concentration of the activator itself.

Once established, this low-level activator gradient could direct the regeneration of a two-zone prestalk-prespore pattern in any isolated fragment of prestalk or prespore tissue. In any fragment lacking prestalk (activated) tissue, the gradient would ensure that the first



cells to become activated would be those at the front. In any fragment which lacked prespore (inhibited) tissue, the gradient would ensure that the first inhibited cells appeared at the rear. In either case the two-zoned pattern thus formed would undergo further regulation until the correct proportion of activated to inhibited tissue had been attained.

The initial formation of the prestalk-prespore pattern can be explained in essentially the same fashion, as a process controlled by an activator concentration gradient previously established by sorting-out. According to this explanation, pattern formation would appear similar to pattern regeneration, and one would not expect to observe sorting of prestalk and prespore cells during the process. However, it is also possible to envision the activator-inhibitor interaction operating in the mass of aggregating cells *before* sorting-out has occurred. Under these circumstances, the activator-inhibitor interaction would initially produce a random-appearing mixture of prestalk and prespore cells. The prestalk cells would be those with higher, and the prespore cells those with lower basic activator production levels. These would become arranged into a two-zoned pattern during the subsequent sorting according to basic production level. In this case the prestalk-prespore pattern would appear to have been formed by sorting-out. If one supposes that one or the other of these modes of pattern formation prevails, according to circumstances, it is possible to account for the fact that that sorting-out of prestalk and prespore cells has been observed in some studies of pattern formation but not in others (see *Mode of Formation and Regulation of the Prestalk-Prespore Pattern*, above).

The range and shape of a gradient established by sorting-out might vary according to the mix of physiologic types in the population of aggregating cells, and the gradient might in fact contain a step in a slug formed by coaggregation of two distinct cell populations [95]. This presents no difficulty, since these parameters are unimportant in the activator-inhibitor model; the gradient only needs a certain minimum slope to control the polarity and configuration of the prestalk-prespore pattern. The activator-inhibitor model can thus make use of a sorting-out gradient, while other models, such as positional-information models, have much more difficulty doing so.

*Volume Dependence and Allometry.* The activator-inhibitor model can account for the quantitative features of the prestalk-prespore pattern which give difficulties in other models. Any reaction-diffusion model which uses direct negative feedback from the prestalk zone would in

fact be expected to regulate the prestalk-prespore ratio on the basis of volume and not length. The activator-inhibitor model in particular can also explain the positive allometry of the prespore zone. The key to this explanation is the fact that the breadth of the transition zone in the activator pattern is determined by the activator diffusion range and does not vary with the size of the slug. This breadth may be only a small fraction of the length of a large slug, but in a small slug it becomes significant. If one assumes that the cells of the transition zone are all assigned prestalk character, the prestalk region will then be proportionally larger in small slugs, as is in fact observed. The activator would have to have a diffusion range of about six cells to account for the observed allometry. Preliminary studies [51] suggest that this is consistent with all other requirements in the application of the activator-inhibitor model to slime mold slugs, but more extensive work is required to establish this definitively.

*Transplantation Phenomena.* The activator-inhibitor model predicts that several distinctive phenomena should be observable in transplantation experiments. The predictions are most straightforward for experiments in which a small piece of tissue is transplanted into a much larger piece, for under these circumstances the outcome will be determined by the transplant's own activator level and by the inhibitor level prevailing in the host tissue. If, for instance, prestalk tissue is transplanted into the prespore zone of a slug in which the prestalk : prespore ratio is lower than normal, and the inhibitor concentration is thus below the critical level, tissue in the transitional zone surrounding the transplant will tend to become activated. Activation will then spread from the transplant in all directions, and the transplant will appear to 'induce' a prestalk zone. A similar phenomenon of 'prespore induction' should be observable when prespore tissue is transplanted into prestalk tissue in a slug in which the prestalk : prespore ratio has been increased, and the inhibitor concentration is higher than the critical level.

Aside from the effects produced by direct manipulation of the prestalk-prespore ratio, the likelihood of prestalk or prespore induction in transplantation experiments should depend on both the original position of the transplanted tissue (that is, its position while still in the donor), and on the position into which the transplant is put (in the host). The effect of position in the host arises because the inhibitor, which diffuses from the prestalk zone into the prespore zone, is expected to show at least a weak concentration gradient; the inhibitor concentration will slightly exceed the critical level in the front, and

will be slightly below this level in the rear. The effect of the original position results from the fact that the activator concentration is higher in tissue taken from the prestalk zone (activated tissue) than from the prespore (inhibited) zone. A small apparent 'activation gradient' is also expected within each zone as a consequence of the activator basic production gradient.

The fate of induced prestalk or prespore zones is uncertain. One possibility is that their cells would move quickly to the main prestalk and prespore zones due to sorting; in this case the induction phenomenon might be hard to observe. If, however, the formation of the slug tip is a function of prestalk tissue, induced prestalk zones might be stabilized by their formation of accessory tips. The original and final position effects on prestalk induction would then appear as gradients of inhibition of tip formation and of resistance to inhibition. The activator-inhibitor model could thus account for the gradients described by Durston [70].

Since we have already noted that Durston's results can be explained by the stable/labile gradient model (see above) it is worth mentioning that the tip-formation phenomena predicted by this model and those predicted by the activator-inhibitor model differ in detail. In the stable/labile gradient model, both original and final position effects arise from linear gradients, and the levels of these gradients at the front and rear ends of the slug are independent of the overall slug size. In the activator-inhibitor model, however, the activator profile (responsible for the original-position effect) is strongly nonlinear, since it includes a step at the prestalk-prespore boundary. (Durston and Vork [99] appear to have demonstrated such a step.) The inhibitor pattern shows no step, but its levels at the front and rear of the slug should depend on slug size, being more extreme in longer slugs. The models also differ in their predictions of the effect of adding or removing prestalk or prespore tissue if the ends of the slug are not disturbed in the operation. It may thus be possible to build a case in favor of one or the other of these models using transplantation experiments. Transplantation phenomena must of course be interpreted with caution, since the relationship between induction in transplantation experiments and the levels of gradients cannot be entirely straightforward; this is discussed in detail in reference [110].

The specific hypothesis in the activator-inhibitor model of a low-level activator gradient generated by sorting-out could be tested by comparing gradient levels in slugs from strains which normally sort out from one another: The average gradient level should be higher in a slug composed of cells with a high tendency to sort to the front than in one made of cells that tend to sort to the rear.

*Mutants.* A class of mutants specifically diagnostic for the activator-inhibitor model may occur: mutants in which sorting-out is defective and the sorting-out gradient is not formed. Prestalk and prespore tissue in such mutants might be found in a variety of configurations other than the normal two-zone pattern. Since tip formation by prestalk tissue (if this occurs) could make these mutants hard to observe, the activator-inhibitor model does not strongly predict that it should be possible to find them. If found, however, these mutants would be difficult to account for with positional-information models. The additional fact that these mutants should consistently display a normal prestalk-prespore proportion could not be easily explained by cell-contact models.

### *Hybrid Models*

The properties of the cell-contact, positional-information and activator-inhibitor models are summarized in Table 1. These models seem to us to be the simplest ways to account for prestalk-prespore patterning in cellular slime molds, but it is quite possible that none of these models is correct. It is therefore worth pointing out that many of the other pattern formation mechanisms that one might imagine can be brought into the framework of this discussion by considering them as 'hybrids' — models incorporating elements of more than one of the models treated here. This can be a useful way of thinking, since the experimental phenomena predicted by a 'hybrid' model often turn out to be simply related to the phenomena predicted by its 'parents'.

For instance, one might imagine a modified activator-inhibitor model in which the activator does not diffuse from cell to cell at all, activation spreading instead by a contact-mediated cell interaction. Such a mechanism might allow the prestalk-prespore boundary to migrate faster than would be possible were activation mediated by a diffusing substance. According to the modified model one would expect to find transplantation phenomena similar to those predicted by the activator-inhibitor model, but one might also expect to recover mutants with properties related to the cell-contact interaction which would severely disturb patterning even when only a few mutant cells were present.

One might also imagine that a diffusion-reaction system is involved in prestalk-prespore patterning but that this mechanism produces a proportion-regulated gradient rather than the steplike pattern predicted by the activator-inhibitor model. A gradient-interpretation mechanism would then be required to fix the exact position of the prestalk-prespore boundary. Were this the



Table 1. Comparison of three models of prestalk-prespore patterning in cellular slime molds

Mechanism of initial establishment of pattern	Mechanism of pattern regulation	Role of sorting-out in patterning	Explanation of volume dependence and allometry of pattern	Special predictions
I. cell-contact model:				
Positive and negative waves generated by cell-contact interactions	? (None, unless patterning is repeated periodically)	?	?	Severe pattern alterations following transplantations or admixture of small percentages of cells from certain mutants
II. Positional-information model:				
(A) Surface-sheath model:	Migration establishes a gradient in the surface sheath; this 'interpreted' by a gradient-reading mechanism	Changes in migration rate	?	Gradient-reading mutants (show double boundaries when mixed with wild-type cells)
(B) Stable/labile-gradient model:	Interpretation of stable gradient; (establishment of this gradient problematical)	Stable gradient 'follows-up' labile gradient	?	Gradient-reading mutants; certain transplantation properties
III. Activator-inhibitor model:				
Similar to regulation, or initial partitioning into prestalk and prespore cells according to activator basic production level, followed by sorting-out according to this level	Negative feedback brings about appropriate cell-type conversion; spatial coordination due to activator basic production gradient	Establishes activator basic production gradient	Negative feedback mechanism intrinsically volume-dependent; transition zone specified as prestalk	Certain transplantation properties; mutants with multiple prestalk zones

case, one might expect both transplantation phenomena resembling those of the activator-inhibitor model and the 'gradient-reading' mutants normally characteristic of positional-information models. An example of this kind of mechanism (which unfortunately shows little capacity for proportion-regulation) is Lacalli and Harrison's [111] application of Turing's [112] classic diffusion-reaction model to slime mold slugs.

Hybrids between cell-contact models and positional-information models might also be envisioned; here a direct cell-to-cell interaction would give rise, in effect, to positional information, and the positional information would then control cell differentiation [113]. Such a mechanism might well be sensitive to misbehavior on the part of a few cells, but would also be consistent with the existence of 'gradient interpretation' mutants; it would thus have characteristics of both cell-contact and positional-information models.

## Conclusion

The prestalk-prespore pattern in cellular slime molds is an intriguing biologic phenomenon, which can be approached experimentally in many ways. The models we have discussed clearly do not include all possible mechanisms for the formation and regulation of this pattern, but they do represent all three of the major classes of models which have been used to account for biological pattern formation in other systems. It seems reasonable to suppose that the actual mechanism of prestalk-prespore patterning is similar to one of these models, or perhaps incorporates elements of two of them. In any case, the models can serve as a starting-point for many sorts of investigations.

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# Proteases in cellular slime mold development: Evidence for their involvement

(cell differentiation/protein degradation/*Dictyostelium discoideum*/amino acid rescue)

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**ABSTRACT** Protein degradation appears to be essential for normal differentiation in the cellular slime mold *Dictyostelium discoideum*. Several protease inhibitors block normal differentiation, and in most cases this inhibition can be reversed by addition of amino acids. For example, chloroquine, which inhibits slime mold cathepsin B activity, interfered with development by blocking sorocarp formation, and this inhibition was reversed by the addition of amino acids. Tosyllysyl chloromethyl ketone also blocked development, and this inhibition was reversed by simultaneous additions of amino acids and glutathione. Moreover, the addition of antipain and leupeptin delayed sorocarp formation. These results, together with the finding reported earlier that cathepsin B activity is differentially localized in the prestalk–prespore zones of the migrating slugs, suggest that proteolysis might play a regulatory role in cellular slime mold differentiation.

In the life cycle of cellular slime molds the growth phase is separate from the differentiation phase. Aggregation begins only after starvation, probably because of amino acid deprivation, as Marin (1) has shown. It has been known since the work of Gregg *et al.* (2) that during the period of differentiation, when no exogenous sources of energy are taken in, proteins are degraded; the products are presumed to be used as an endogenous source of energy (see also refs. 3–6.) Furthermore, a number of workers have provided direct evidence for proteolysis during development (7–12).

Here we provide further evidence that protease activity might be involved in, and even required for, differentiation. In an earlier study (11) it was shown that the proteolytic activity of cathepsin B was higher in the prestalk than in the prespore zone, which opens up the possibility that it might be playing a role in the control of stalk cell–spore differentiation. Here we support such a premise by showing that not only is it possible to inhibit normal development with various protease inhibitors, but the inhibition can, in some instances, be reversed by adding amino acids. From this comes the hypothesis that proteolysis is not just a means of making energy and amino acids available to the starving cell mass, it is an essential component in the normal development of cellular slime molds.

## MATERIALS AND METHODS

**Strains.** Two strains of *Dictyostelium discoideum* were used: strain NC4 of K. B. Raper and a mutant of this strain, A2, that grows axenically (13).

**Conditions for Growth.** The amoebae of NC4 were grown with *Escherichia coli* B/r on buffered nutrient agar (14). The amoebae of A2 were grown axenically in HL-5 medium in liquid culture (13). Amoebae were harvested during the exponential phase of growth and washed in 100% Bonner's salt solution before use. Cell numbers were estimated by dilution and counting in a hemocytometer.

**Conditions for Differentiation.** A drum technique was used to study cell differentiation (15, 16). A standard drum consists of two close-fitting plastic cylinders, 4.5 cm in diameter, placed

in a 40 × 80 cm crystallizing dish. Between these two cylinders is a dialysis membrane (catalog no. 3787-D52, Arthur H. Thomas), pretreated by boiling for 5 min in 1 mM ethylenediaminetetraacetic acid (EDTA) and then washed in distilled water. Amoebae were placed on the upper side of the dialysis membrane and the dish was filled with buffer solution just up to the lower surface of the membrane. Under these conditions  $3 \times 10^7$  cells formed a monolayer on the membrane and sorocarps appeared at about 24 hr.

Because of the rich nutrients available in the amino acid “rescue” experiments, all materials and glassware, including the 2% Bonner salt solution for harvesting amoebae, were either autoclaved or filter-sterilized (Millipore SX HA 025 OS sterile Swinnex 0.45  $\mu$ m, 25 mm) before use. A large drum of 4.5-cm inner diameter contained  $4.2 \times 10^7$  amoebae, with 35 ml of solution in the glass dish. A smaller drum of 1.2-cm inner diameter contained  $3 \times 10^6$  amoebae, with 3 ml of solution in the glass dish. (Small drums were used when inhibitors were available only in small quantities.) The dishes were covered with glass covers and autoclaved.

**Inhibitors and “Rescuers.”** The buffer to which compounds were added was 16.7 mM Sorensen phosphate, pH 6.0 ( $\text{KH}_2\text{PO}_4$ , 7.94 g;  $\text{Na}_2\text{HPO}_4$ , 1.12 g;  $\text{H}_2\text{O}$ , 1000 ml; then 1:4 dilution with water) containing 2% Bonner's salt solution. Inhibitors were chloroquine (diphosphate salt, Sigma), quinine (hydrochloride, Sigma), tosyllysyl chloromethyl ketone (Tos-LysCH<sub>2</sub>Cl; hydrochloride, Sigma), pepstatin, leupeptin, and antipain (the last three from H. Umezawa). Rescue experiments were attempted with the following: L-leucine (Nutritional Biochemicals), glucose (Difco dextrose), glutathione (reduced form, Sigma), and casamino acids (Difco vitamin-free casamino acids; Difco vitamin assay casamino acids). The normal concentration used was 1% (wt/vol) for casamino acids; concentrations for other compounds are noted in *Results*.

**Assays for Protein Content and Cathepsin Activities.** Cell samples of different developmental stages were harvested from the drums in 2 ml of water, frozen in an ethanol/dry ice bath immediately, and stored at  $-20^\circ\text{C}$  until use. Thawed samples were sonicated and aliquots were taken to measure the protein content (17) and cathepsin activities. For cathepsin D assay, the reaction mixture contained 0.25 ml of casein (4% wt/vol in 0.05 M citrate buffer, pH 2.5), 0.5 ml of cell extract, and 0.5 ml of 0.1 M citrate buffer, pH 2.5. After 50-min incubation at  $37^\circ\text{C}$ , the reaction was terminated by 1 ml of 10% trichloroacetic acid, and the absorbance of acid-soluble products was read at 280 nm. For cathepsin B assay, the reaction mixture contained 0.7 ml of 0.1 M sodium acetate buffer at pH 5.5, 0.1 ml of activators (dithiothreitol and EDTA, 4 mM each),  $\alpha$ -N-benzoyl-DL-arginine *p*-nitroanilide (Sigma, 10 mg/ml in dimethyl sulfoxide),

Abbreviation: TosLysCH<sub>2</sub>Cl, tosyllysyl chloromethyl ketone.

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and 0.5 ml of cell extract. After 5-hr incubation at 37°C, samples were boiled and centrifuged, and absorbance at 410 nm was measured.

**Assay for Intracellular Amino Acid Pool.** NC4 amoebae were put on drums (3 × 10<sup>7</sup> cells per drum) with and without 2.5 mM chloroquine. At 14 hr of development, 6 drums each from drug treatment and control were harvested into 4 ml of cold water and washed once (7000 rpm, 10 min, Sorvall SS-34 rotor). To each sample 3 ml of cold 5% trichloroacetic acid was added at 4°C for 8 hr. Centrifugation (18,000 rpm, 10 min) gave an acid-soluble fraction. Ether was used to extract the acid. Aqueous fractions were dried on a hot plate (slide warmer, Precision Scientific, Chicago, IL) and assayed for amino acids with an amino acid analyzer (Beckman model 120B) (18).

**Assays for Actinomycete Protease Inhibitors.** Partially purified proteases from A2 amoebae were used to study inhibitor effects. A hemoglobin-agarose affinity column, made according to Smith and Turk (19), was chosen for cathepsin D; an α-N-benzoyl-L-argininamide-agarose column, after Ogino and Nakashima (20), was used for cathepsin B. Details for partial purification are presented in Ref. 21. For the partially purified enzyme fractions from affinity columns, hydrolysis of Azocoll (Calbiochem) at pH 5.5 with dithiothreitol/EDTA in the presence and absence of pepstatin, leupeptin, and antipain was used to test for cathepsin B; hydrolysis of hemoglobin (Worthington) at pH 2.55 was used to test for cathepsin D. Pepstatin was first dissolved in methanol, then transferred to dilute NaOH, and then titrated back to pH 7. Leupeptin and antipain were water soluble.

RESULTS

**Effects of Chloroquine on Development.** Membrane drums were used to test the effects of protease inhibitors on *D. discoideum* development. The amoebae were deposited on the surface of a dialysis membrane lying over a buffered salt solution. Various compounds were tested for their effects on cell differentiation by placing them in the buffer solution.

Chloroquine was shown previously to inhibit cathepsin B activity *in vitro* in *D. discoideum* (11). In the presence of 2.5 mM chloroquine, amoebae aggregated but further morphogenetic processes were inhibited. At the end of development the control cells differentiated into mature sorocarps (Fig. 1A) whereas the drug-treated cells merely formed aggregation clumps (Fig. 1B). Chloroquine inhibition of development was stage-specific; its effects were seen only when the cells were exposed to chloroquine before the migration stage. [The fact that there is a slime sheath in the slug acting as a possible permeability barrier should be noted (22).] Moreover, chloroquine inhibition was reversible; after the drug had been removed, sorocarp development continued, following an initial time delay.

Protein content and protease activities were measured at different time periods for cells on the drums with or without

chloroquine. Drug-treated cells consistently contained more protein than the corresponding control cells: chloroquine reduces the rate of protein degradation (Fig. 2A). When these cells were tested for cathepsin activities, chloroquine-treated cells had higher cathepsin D (Fig. 2B) and cathepsin B (Fig. 2C) activities than control cells. These measurements were made by washing the cells, sonicating them, and taking samples for protease assays. Superficially it seems contradictory that chloroquine-treated cells would have higher cathepsin B activity than control cells, because chloroquine inhibits cathepsin B activity *in vitro* (11). However, chloroquine is known to raise the pH of the lysosomes *in vivo* (23), and this may influence the activities of cathepsins and other lysosomal enzymes. Thus one possible explanation would be that the cathepsins themselves, being proteins with catalytic activities, had their own degradation delayed.

If chloroquine really inhibits protein degradation in *D. discoideum* and protein synthesis is always present during development, one would expect exhaustion of the intracellular free amino acid pools in chloroquine-treated cells. As seen in Table 1, when the control reached the slug stage, free amino acid pools were lower in treated samples than in the controls. Chloroquine-treated cells retained only an average of 29% of the free amino acid pools of the control cells.

In order to show that the inhibition of proteolysis is indeed causing the blocking of morphogenesis and differentiation, amino acids were added along with chloroquine to see if they would counter the effect of the drug. Cells exposed to 0.75% (wt/vol) casamino acids with chloroquine (2.5 mM) produced normal sorocarps (Fig. 3B), as did the controls with casamino acids alone. From this experiment it is apparent that the casamino acids are able to reverse or rescue the amoebae from the inhibitory effects on development of chloroquine.

Apparently it is not just a matter of supplying energy, because the addition of glucose (20 mM) did not reverse the effect of chloroquine (Fig. 3C). Similarly, addition of a single amino acid, leucine (10 mM), did not reverse the effect of chloroquine (Fig. 3D). Only a mixture of amino acids, which would mimic the products of protein degradation, could restore the inhibition of development of chloroquine.

**Effects of Other Protease Inhibitors on Development.** In an earlier publication we demonstrated that TosLysCH<sub>2</sub>Cl can inhibit cathepsin B activity in crude cell extracts of *D. discoideum* (11). Consequently it would be of interest to check the effects of this inhibitor on development. TosLysCH<sub>2</sub>Cl at 3 mM completely blocked development (Fig. 4A). In contrast to chloroquine-treated cells, TosLysCH<sub>2</sub>Cl-treated cells did not even form clumps. When these treated cells were supplied with casamino acids (1%, wt/vol), development continued to cell clumps (Fig. 4B). When cells treated with TosLysCH<sub>2</sub>Cl and

Table 1. Free amino acid pools in *D. discoideum*

Amino acid	Pool, nmol/2 × 10 <sup>8</sup> cells	
	Control, 14-hr slugs	Chloroquine, 2.5 mM, 14-hr clumps
Aspartic acid	5.0	2.5
Glutamic acid	26.2	11.2
Glycine	20.1	5.8
Alanine	21.9	4.0
Valine	3.3	Trace
Isoleucine	4.4	0.9
Leucine	3.1	0.6
Phenylalanine	1.5	Trace

Trichloroacetic acid-soluble fractions from drug-treated and control samples were assayed for free amino acid pools.

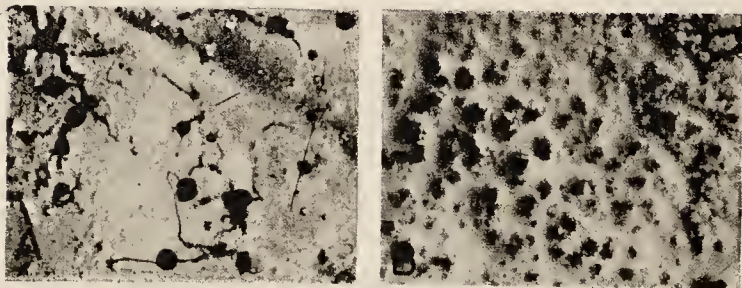


FIG. 1. Inhibition of *D. discoideum* A2 development by chloroquine. (A) Buffer control; (B) 2.5 mM chloroquine. Photographs were taken after 25 hr of development. (×9.)



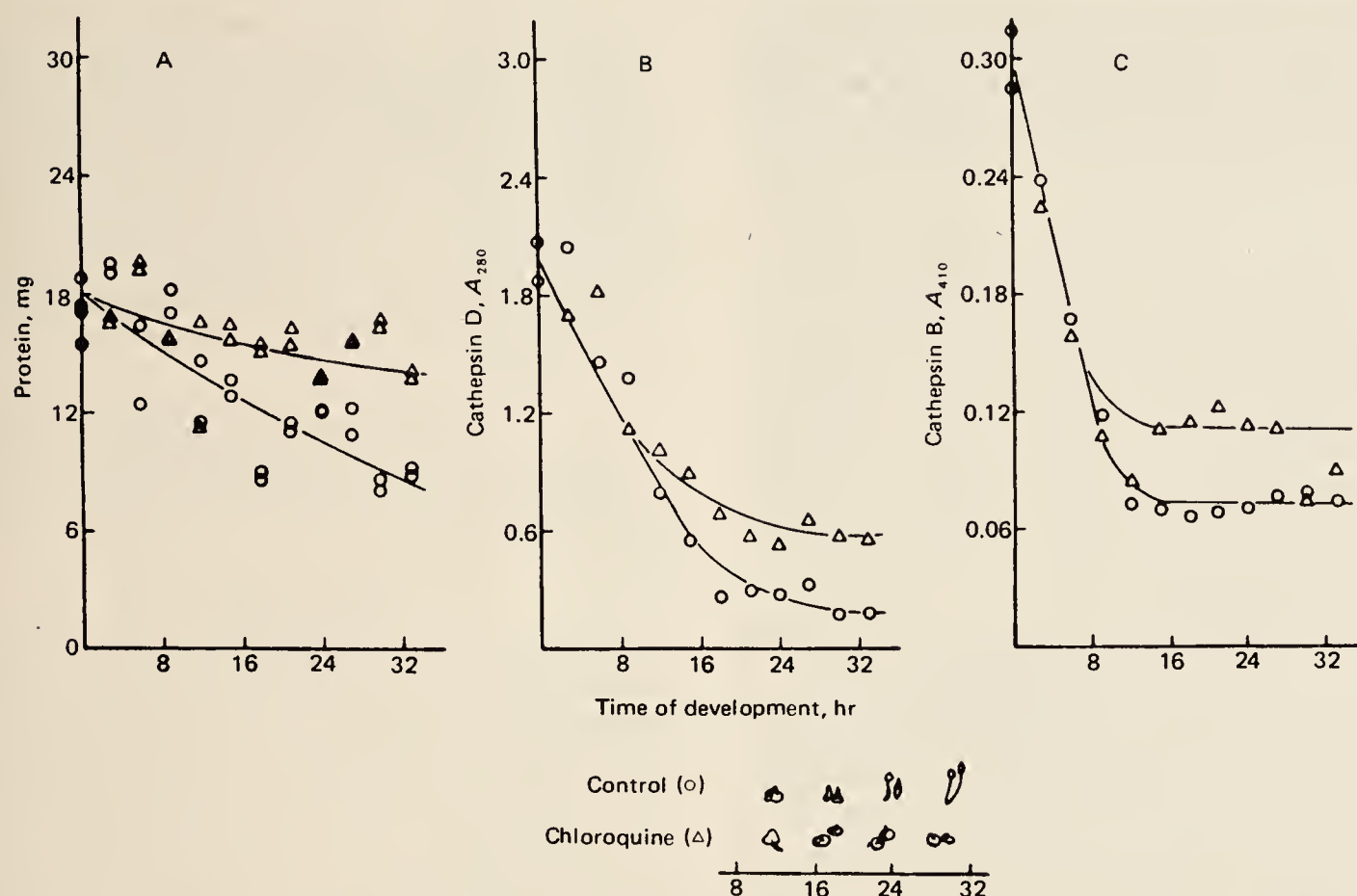


FIG. 2. Changes in protein and cathepsin activities in *D. discoideum* NC4 in the presence of 1 mM chloroquine ( $\Delta$ ) or its absence (O). All assays are given per  $3 \times 10^7$  cells. (A) Protein content, assayed by the Lowry method (17); (B) cathepsin D activity, assayed by casein hydrolysis as  $A_{280}$ ; (C) cathepsin B activity, assayed by benzoylarginine *p*-nitroanilide hydrolysis as  $A_{410}$ . The x axis is time of cell differentiation; the corresponding morphologies have also been sketched.

casamino acids were, in addition, exposed to glutathione (2 mM), they differentiated into normal fruiting bodies (Fig. 4D). The controls showed that glutathione itself had no effect on myxamoebae differentiation (data not shown), nor did it alter the inhibitory effect of TosLysCH<sub>2</sub>Cl (Fig. 4C). It is possible, as suggested by Penn *et al.* (24), that TosLysCH<sub>2</sub>Cl is acting intracellularly by alkylating compounds with free —SH groups such as reduced glutathione.

Quinine is a compound related to chloroquine, and its effect on *Dictyostelium* development is similar. Cells formed clumps and tiny slugs in the presence of 2.5 mM quinine, and this de-

velopmental inhibition could be reversed by the addition of 1% (wt/vol) casamino acids (data not shown).

**Effects of Microbial Protease Inhibitors on Partially Purified *Dictyostelium* Proteases *In Vitro*.** Umezawa and his associates have isolated protease inhibitors from culture filtrates of actinomycetes and have used these compounds to characterize the proteolytic enzymes (25). We had previously found two cathepsin activities in *D. discoideum* crude cell extracts

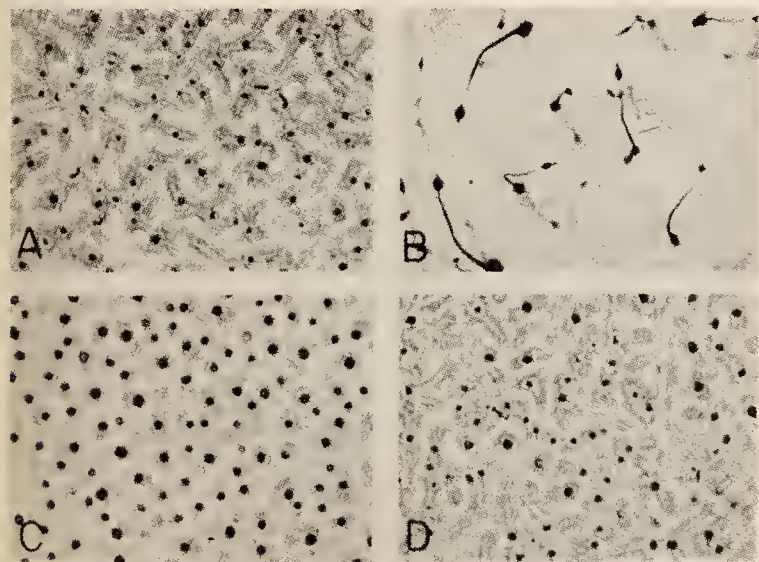


FIG. 3. "Rescue" attempts with chloroquine inhibition of *D. discoideum* NC4 differentiation. (A) Chloroquine at 2.5 mM; (B) chloroquine and 0.75% casamino acids; (C) chloroquine and 20 mM glucose; (D) chloroquine and 10 mM leucine. (X8.)

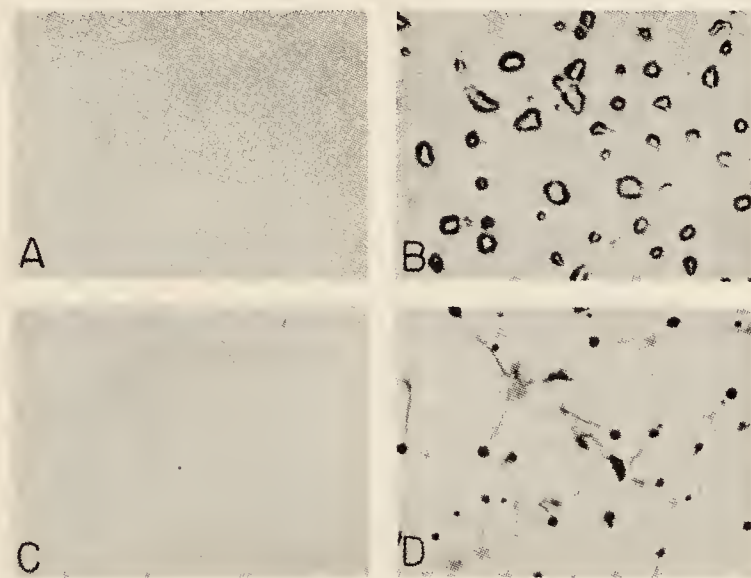


FIG. 4. Inhibition of *D. discoideum* NC4 by TosLysCH<sub>2</sub>Cl. (A) TosLysCH<sub>2</sub>Cl at 3 mM; (B) TosLysCH<sub>2</sub>Cl and 1% casamino acids; (C) TosLysCH<sub>2</sub>Cl and 2 mM glutathione; (D) TosLysCH<sub>2</sub>Cl and casamino acids and glutathione. All three compounds were mixed in the buffer before the addition of amoebae. All were 28-hr samples. (X8.)



(11), and we extended our studies by using microbial protease inhibitors together with partially purified myxamoebae enzyme fractions. As shown in Fig. 5A, the activity of the cathepsin D-like enzyme eluted from a hemoglobin-Sepharose column was inhibited up to about 60% by pepstatin. Moreover, the activity of the residual enzyme was unaffected by pepstatin even though the concentration of inhibitor was increased by three orders of magnitude. This suggests that a pepstatin-insensitive acid protease may be present together with the cathepsin D-like protease in our partially purified enzyme fraction. Furthermore, both leupeptin and antipain had no effect on this cathepsin D-like activity (Fig. 5B and C). On the other hand, both leupeptin and antipain decreased by 90% the cathepsin B-like enzyme activity eluted from a benzoylargininamide-Sepharose column, whereas pepstatin had no effect on activity of this enzyme (Fig. 5). Thus we have evidence that both cathepsin D and cathepsin B are found in *D. discoideum* and that these enzymes can be separated from one another by affinity chromatography.

#### Effects of Microbial Protease Inhibitors on Development.

We then tested leupeptin and antipain for their effects on cell differentiation. Pepstatin was not attempted because of its poor solubility in aqueous buffer and we needed to use concentrations that were orders of magnitude greater than required for the inhibition of enzyme activities *in vitro*. At 2.5 mM, both leupeptin and antipain delayed sorocarp development. When sorocarps appeared in the control, leupeptin-treated cells showed slugs and early culminating cell masses (data not shown). Antipain-treated cells differentiated even more slowly and were at late aggregation and early slug stage (Fig. 6B) when the controls became sorocarps (Fig. 6A). However, after the delays, sorocarps were formed in these leupeptin- and anti-

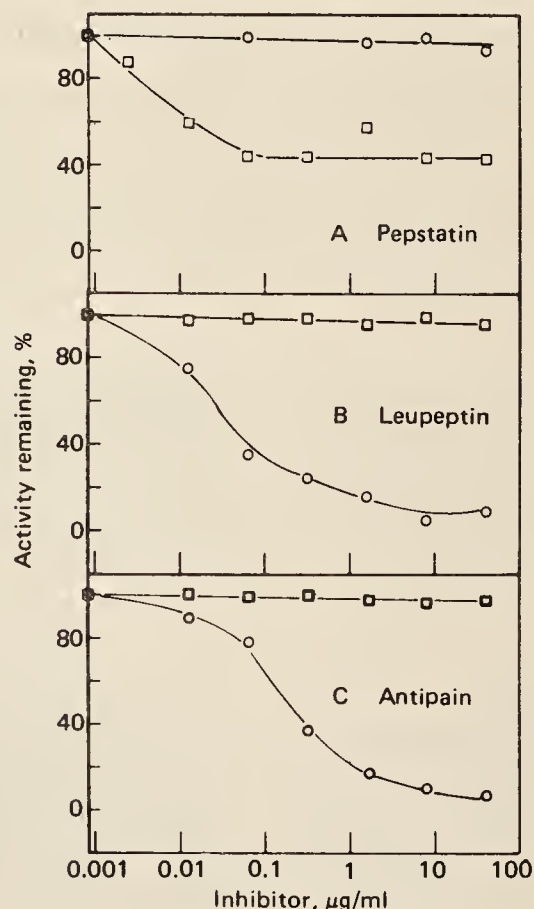


FIG. 5. Inhibition of *D. discoideum* protease activities by actinomycete protease inhibitors. (A) Pepstatin; (B) leupeptin; (C) antipain. The cathepsin D ( $\square$ ) assay was hemoglobin hydrolysis at pH 2.55; the cathepsin B ( $\circ$ ) assay was Azocoll hydrolysis at pH 5.5 in the presence of dithiothreitol and EDTA. At zero inhibitor concentration, cathepsin is considered to possess 100% enzymatic activity.



FIG. 6. Delay of *D. discoideum* NC4 cell differentiation by antipain. (A) Buffer control and (B) 2.5 mM antipain, both at 24 hr of development. ( $\times 8$ .)

pain-treated samples. These effects of actinomycete protease inhibitors on *Dictyostelium* development also suggested that protease activity is required for differentiation, at least in the sense of finishing the program within a normal period of time.

## DISCUSSION

We have shown that protease inhibitors can delay or inhibit cell differentiation in *D. discoideum*. In a number of cases the inhibitions can be reversed by the simultaneous addition of amino acid mixtures. Because a large number of new enzymes and other proteins are synthesized during development (26) in spite of the net loss in total protein content (4), the most direct effect of protease inhibitors may be in reducing the amino acids required for protein synthesis *de novo*. Our results are consistent with the view that proteolysis is a necessary step in the differentiation of the cellular slime mold and that the responsible proteases are cathepsin-like. Before returning to this main point, let us briefly review the previous work on proteolysis in the cellular slime molds and show how it relates to the work presented here.

**Evidence for Protease Activity.** Sussman and Sussman (7) found acid protease activity in *D. discoideum*, and Wiener and Ashworth (8) reported its possible localization in the lysosomes. Gustafson and Thon (12) suggested the presence of phosphoryl moieties in a *Dictyostelium* protease that they characterized and named proteinase I. Evidence was presented by Rossmanno *et al.* (10) for the secretion of acid protease activity into the medium during the aggregation stage. The appearance of one new protease band at the culmination stage was detected electrophoretically by North and Harwood (27).

**Protease Inhibitors.** The inhibition of acid protease by protease inhibitors was studied by North (28), who showed that diazoacetylnorleucine methyl ester, but not pepstatin or 1,2-epoxy-3-(*p*-nitrophenoxy)propane, was effective in inhibiting the *Dictyostelium* acid protease. However, both diazoacetylnorleucine methyl ester and pepstatin can inhibit *Dictyostelium* cathepsin D activity under our conditions (11). This difference may be due to the substrates used for the enzyme assay (hide powder azure was used by North and hemoglobin by us). Furthermore, we have characterized a second protease, which is cathepsin B-like, that can be separated from the cathepsin D-like protease. *Dictyostelium* cathepsin D is inhibited by pepstatin, diazoacetylnorleucine methyl ester, and phenylpyruvic acid; *Dictyostelium* cathepsin B is inhibited by leupeptin, antipain, iodoacetate, iodoacetamide, TosLysCH<sub>2</sub>Cl, chloroquine, and a crude egg white extract (presumably containing the papain inhibitor that also inhibits cathepsin B). Moreover, *Dictyostelium* cathepsin B can inactivate rabbit muscle aldolase at pH 6 whereas cathepsin D cannot (unpublished data), and this aldolase inactivation has been reported as a characteristic for cathepsin B (29).

**Rescue Experiments.** The fact that we were able to reverse



the effects of certain protease inhibitors by adding a mixture of amino acids seems, at first glance, to conflict with the experiments of Marin (1), who showed that amino acid starvation was the specific stimulus in initiating development in *D. discoideum*. Yet our experiments showed normal development in the presence of vitamin-free casamino acids (1%, wt/vol) in buffer. This discrepancy can probably be accounted for by differences in concentration. With 2% (wt/vol) casamino acids the amoebae did not differentiate (data not shown), thus giving results similar to those of Marin. (He used a concentration of 0.5 mg/ml for each of his amino acids, but it should be noted that his conditions for differentiation were very different from ours because he used submerged cultures.)

**Role of Proteolytic Activity in Development.** It had always been assumed from the early work of Gregg *et al.* (2) and others that the sole purpose of the degradation of protein was to supply energy for the developing, starved cells. This was thought to be the natural result of separating the growth phase from the developmental phase of the life cycle. Hames and Ashworth (5) questioned this assumption and reported the same rate of proteolysis regardless of whether or not the amoebae were starved of glucose. In the light of more recent work, including the experiments presented here, we can now interpret Hames and Ashworth's pioneering finding by assuming that the proteolysis may be entirely or partially involved in specific developmental or differentiation processes.

In some cases previously described it has been suggested that the proteolysis itself is important to achieve a specific developmental step. For instance, O'Day (9) proposed that the acid proteases in a related species, *Polysphondylium pallidum*, played a specific role in the removal of microcyst wall during its germination. In *D. discoideum*, Rossomando *et al.* (10) postulated that extracellular proteolytic activity may release cell membrane components to facilitate amoebae migration and aggregation. The curbing of proteases has been suggested by Wright and Thomas (30) to be the cause of developmental accumulation of enzymes at the stage of culmination. Evidence supporting this view has come from DeToma *et al.* (31), who found that the *in vitro* stability of the enzyme UDP-glucose pyrophosphorylase was increased by the protease inhibitor TosLysCH<sub>2</sub>Cl.

Another way in which proteolysis might play a significant role is in the control of development. The fact that there is a differential distribution of a particular protease in prespore and prestalk cells (11), and the fact reported here that protease inhibitors that retard or block development can be reversed with mixtures of amino acids, suggest that in some interesting way proteases may be playing a key role in differentiation. (This hypothesis is crudely analogous to the situation in vertebrates, in which cell death plays a central role in certain stages of development, such as in digit formation.) It is not clear how proteolysis might control differentiation, although certainly one possibility is that it has to do with the supply (perhaps a differential supply) of amino acids. Even the end product of protein and amino acid degradation, ammonia, may play a regulatory role in development, as Sussman and Schindler (32) and others have suggested.

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**Evidence for aggregation center  
induction by the ionophore A23187  
in the cellular slime mold *Polysphondylium  
violaceum***

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*Summary.* A thin glass fiber coated with the ionophore A23187 placed among preaggregation amoebae of *Polysphondylium violaceum* will induce numerous ag-



*Fig. 1.* *Polysphondylium violaceum* amoebae aggregating near A23187-coated glass fibers. (a) 1% std salts; (b) 1% std salts plus 1 mM  $\text{MnCl}_2$ . The random

distribution of aggregation centers is also seen when the amoebae aggregate near an uncoated control fiber. Bars, 0.5 mm.



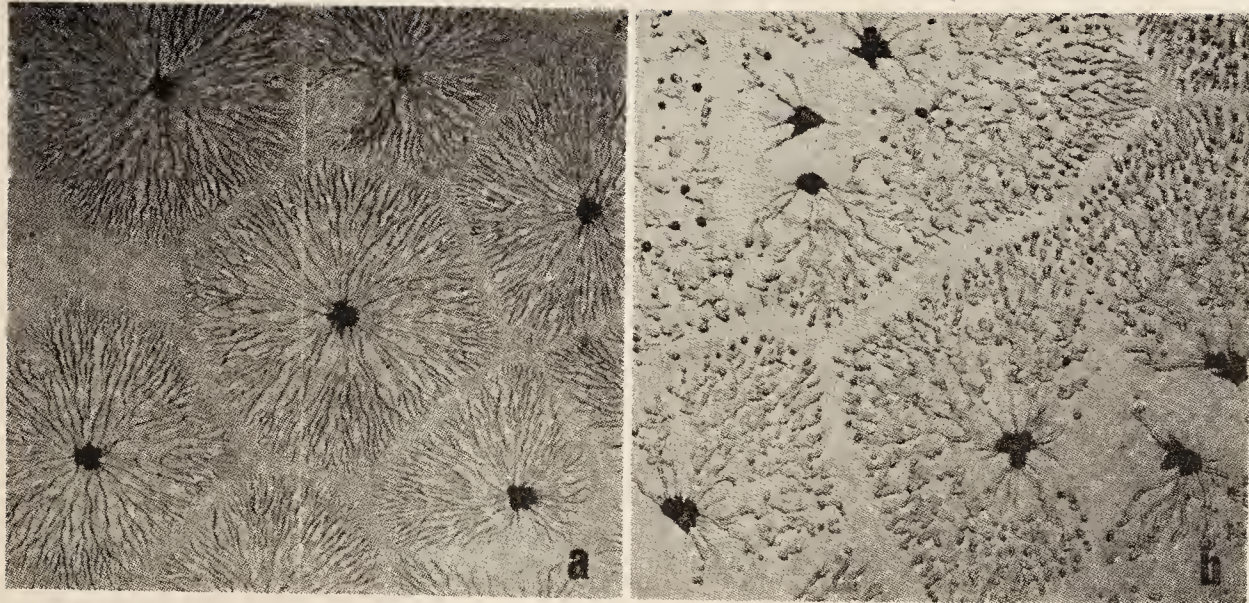


Fig. 2. Aggregation of *Polysphondylium violaceum* amoebae. (a) 1% std salts; (b) 1% std salts plus

$5 \times 10^{-7}$  M A23187. Note the secondary centers in the streams.

gregation centers along the fiber. Both calcium and magnesium ions appear to be involved in this induction. A23187 is also seen to disrupt the normal cell streaming process by producing secondary centers in the aggregation streams.

With the brown alga *Pelvetia fastigiata* Robinson & Cone [1] showed that it is possible to induce rhizoid polarity by placing the fertilized eggs near a fine glass fiber coated with the ionophore A23187; the rhizoids tended to form on the side nearest the fiber. Since the ionophore is quite insoluble in water, a stable ionophore gradient is created near the fiber. This gradient in turn presumably produces divalent cation gradients in the cells in the vicinity of the fibers, and possibly alters the absolute intracellular divalent cation concentrations also.

In an attempt to determine whether or not an A23187 gradient can effect the development of cellular slime molds, glass fibers coated with various ionophores were placed in culture dishes containing different species. In one instance a particularly dramatic result is produced: in preaggrega-

tion amoebae of *Polysphondylium violaceum*, aggregation centers are induced in great numbers along an A23187-coated fiber. In this paper we will describe the result, and further experiments which suggest a complex role for both calcium and magnesium ions in the induction of aggregation centers.

#### Materials and Methods

The following strains of slime molds were used: *Polysphondylium violaceum* No. 1, *Dictyostelium discoideum* NC4, and a variant strain, P4. These were all grown on *Escherichia coli* B/r on nutrient agar (buffered 1% peptone and 1% glucose on 2% agar) and harvested during the vegetative stage. The amoebae were washed three times by centrifugation (5 min at 150 g) in 1% salt solution [2]. They were then resuspended in 1% salt solution and approx.  $5 \times 10^7$  cells were placed on the non-nutrient agar in Petri dishes to which 5 ml of additional 1% salt solution was added. After the cells had settled on the agar surface (15 min) the liquid was carefully poured off, the ionophore-coated fibers were placed on the agar, and the surface was then dried in a laminar flow hood. The plates were incubated at 22°C.

The ionophore-covered glass fibers were prepared by pulling the tips of Pasteur pipettes to a diameter of 50–75  $\mu$ m. They were washed in ethanol and placed on a glass slide. The fibers were coated with 3 mM ionophore (in 100% ethanol) solutions by running the liquid along the fiber with a 33-gauge Hamilton syringe. The ionophores used were A23187, Nigericin



Table 1. *The effect of varying ionic and pH conditions on the induction of centers in P. violaceum by fibers coated with various ionophores*

All Petri dishes contain 2% agar and 1% standard salts

Ionophore on fibers	Conditions	Center induction
A23187	10 mM MES, pH 5	Weak
	10 mM MES, pH 6	+
	10 mM MOPS, pH 7	+
	10 mM MOPS, pH 8	+
	10 mM TRIS, pH 9	+
	10 <sup>-5</sup> M MnCl <sub>2</sub>	+
	10 <sup>-4</sup> M MnCl <sub>2</sub>	+
	10 <sup>-3</sup> M MnCl <sub>2</sub>	—
	5 × 10 <sup>-3</sup> M MnCl <sub>2</sub>	—
Nigericin	10 mM MES, pH 5	—
	10 mM MES, pH 6	—
	10 mM MOPS, pH 7	—
	10 mM MOPS, pH 8	—
	10 mM TRIS, pH 9	—
A23187	1 mM MnCl <sub>2</sub> +:	
	5 mM MgCl <sub>2</sub>	—
	0.01 M MgCl <sub>2</sub>	—
	0.02 M MgCl <sub>2</sub>	+
	0.04 M MgCl <sub>2</sub>	+
	0.1 M MgCl <sub>2</sub>	+
	5 mM CaCl <sub>2</sub>	—
	0.01 M CaCl <sub>2</sub>	—
	0.02 M CaCl <sub>2</sub>	—
	0.04 M CaCl <sub>2</sub>	—
	0.01 M CaCl <sub>2</sub>	+
Gramicidin	No additions	—
Valinomycin	No additions	—

(both kindly donated by Dr Robert J. Hosley of Eli Lilly and Co.), Gramicidin (kindly supplied by Dr Frank Harold) and Valinomycin (Sigma). MES, MOPS, and TRIS buffers were from Sigma Chemical Co.

Agar containing A23187 was prepared by autoclaving 2% agar, allowing it to cool to 45°C, and then quickly stirring in the proper amount of 5 mM A23187 stock solution to produce an A23187 concentration of 5 × 10<sup>-7</sup> M.

Results

*The effect of A23187.* When glass fibers covered with A23187 are placed among vegetative amoebae of *Polysphondylium violaceum* on 2% agar containing 1% stand-

ard salts [2] numerous aggregation centers appear alongside the fiber after 6–12 h of incubation (fig. 1a). Control fibers without any ionophore, or A23187 fibers placed on agar containing 1% standard salts and 1 mM MnCl<sub>2</sub> show a random distribution of aggregation centers (fig. 1b). No effects were seen with other species or other ionophores except when A23187 fibers were placed among *D. discoideum* NC4 or P4 amoebae. They showed a faint suggestion of the effect seen so dramatically in *Polysphondylium violaceum*.

In an attempt to understand the basis for the pattern seen in fig. 1 three possibilities were tested: (1) that chemotaxis occurred toward the fiber due to the cation flux induced by the ionophore; (2) that founder cell induction occurred; and (3) that, in addition, aggregation centers were induced in cell streams. As we shall see, the evidence points to the latter two possibilities.

(1) In careful microscopic observation of cells near the fiber no systematic orientation of preaggregative amoebae toward the fiber was seen. Experiments were also run using the Zigmund chamber [3] for chemotaxis with 1% standard salts in each well plus an A23187 fiber in one well to create an A23187 gradient similar to the one produced on agar plates, but again no orientation of aggregation-competent amoebae was seen. Finally, if cells were placed in a small drop on agar, the movement of cells out of the drop was not affected by placing an A23187 fiber nearby. None of these experiments rule out the possibility of an ion gradient producing an orienting effect on aggregating amoebae, but they do rule out the possibility that the pattern seen in fig. 1a is primarily due to chemotaxis.

(2) There is clear evidence that *P. violaceum* founder cells are induced to begin forming centers along the fiber. This may

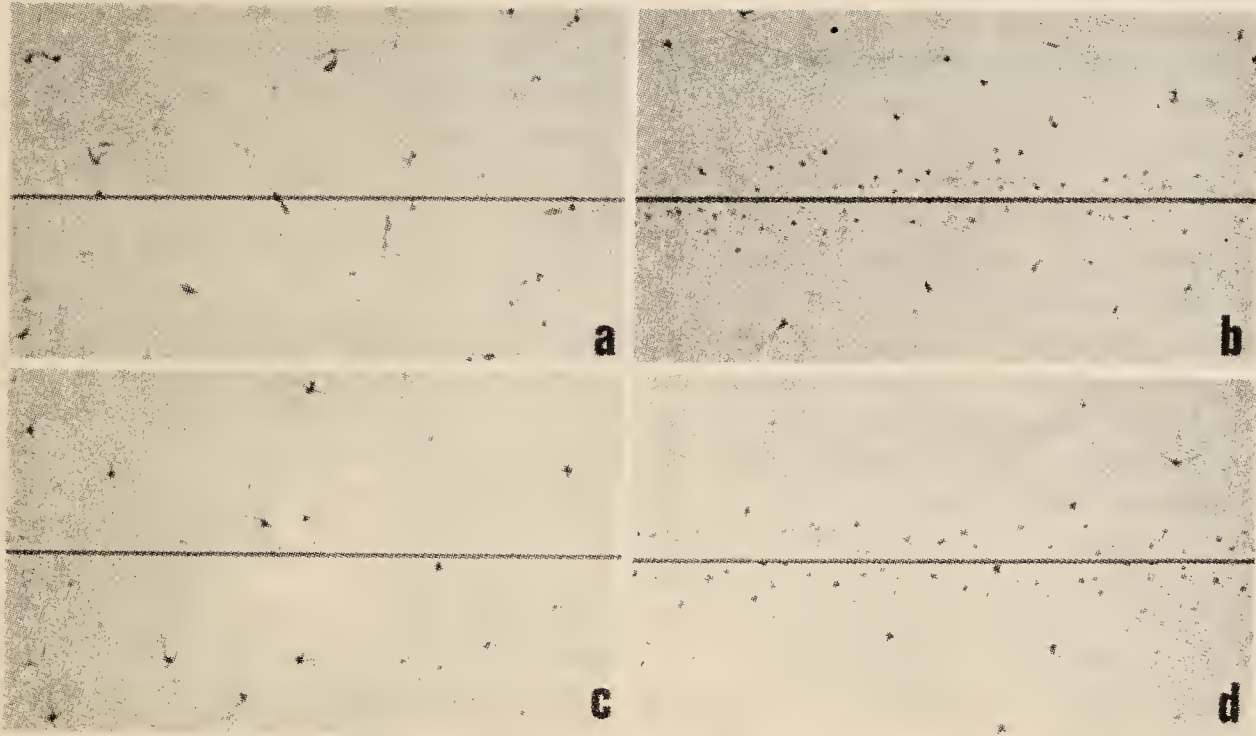


Fig. 3. The effect of high concentrations of  $\text{CaCl}_2$  and  $\text{MgCl}_2$  on *P. violaceum* amoebae aggregating on agar containing 1% std salts and 1 mM  $\text{MnCl}_2$ . (a) 0.1 M

$\text{MgCl}_2$ , uncoated fiber; (b) 0.1 M  $\text{MgCl}_2$ , A23187-coated fiber; (c) 0.1 M  $\text{CaCl}_2$  uncoated fiber; (d) 0.1 M  $\text{CaCl}_2$ , A23187-coated fiber.

be seen by watching for the first centers to be formed on a test plate. While initial centers may form anywhere on the plate, at about the same time a large number of centers will form close to the fibers (cf fig. 1a, b).

(3) It is also clear that the ionophore induces secondary centers in streams. This can be seen in streams which form parallel to the fiber and subsequently break up into centers as is evident in portions of fig. 1. Also, if *P. violaceum* amoebae are plated on agar containing ionophore (see Methods) there is an increase in the number of streams that break up, forming secondary centers (fig. 2).

This latter observation raises the question of whether a cation flux across the cells near the fiber is necessary for the effect, or whether an actual change in intracellular cation concentrations is responsible for inducing the centers. At the moment the

question remains unresolved; perhaps both gradients and critical concentrations play a part.

It is important to note that in the presence of A23187, centers often do not develop into fruiting bodies but remain in small rounded capsules. However, some time after being transferred to a fresh plate without A23187 they will resume development and fruit normally. Also, the ionophore tends to induce numerous torus-shaped groups of aggregating amoebae.

*How the ionophore works.* A23187 is a monocarboxylic cation-proton exchange ionophore which facilitates an electroneutral exchange of cations for protons across most lipid bilayers [4, 5, 6]. In model membrane studies, the ionophore is found to extract cations with the following specificity:  $\text{Mn}^{2+}(210) > \text{Ca}^{2+}(2.6) > \text{Mg}^{2+}(1) > \text{Sr}^{2+}(0.012) > \text{Ba}^{2+}$  (binding ratios in parentheses) [5].



Since the action of A23187 is quite complex, a number of experiments were performed to try to determine which aspect of the ionophore produces the effect in fig. 1a (table 1). Nigericin, which is also a cation-proton exchange ionophore was shown to have no effect on *P. violaceum* development in a pH range of 5–9 when tested with the fiber assay. This seems to suggest that a specific cation transport, and not proton transport is the key to the induction effect. The lack of effect seen with Nigericin, Valinomycin, and Gramicidin fibers suggests that the induction is caused by a divalent cation flux rather than by  $\text{Na}^+$  or  $\text{K}^+$  (A23187 transports these monovalent cations in very small amounts).

When  $\text{MnCl}_2$  was added to the agar in concentrations of 1 mM or above, no center inducing effect was seen near A23187 fibers, though the amoebae still aggregated in a completely normal fashion. Apparently, in the presence of excess  $\text{Mn}^{2+}$  not enough of the crucial divalent cations are transported to produce aggregation center induction. To determine if the ion producing the effect was specifically  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  the basic fiber experiment was run on 1 mM  $\text{MnCl}_2$  agar to which a range of concentrations of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  were added (table 1). When 0.02 M  $\text{MgCl}_2$  was added, center induction was again seen despite the presence of the manganese, while no effect was seen on plates containing less than 0.1 M of calcium. When the experiment was run with 0.1 M  $\text{Mg}^{2+}$  or 0.1 M  $\text{Ca}^{2+}$  an identical effect was seen: although the high salt was toxic to many of the cells, the A23187 fibers induced center formation in nearby surviving cells (fig. 3).

### Discussion

From the experiments described here it is clear that calcium and magnesium are both

involved in the induction of aggregation centers by the ionophore A23187. Since aggregation centers begin by the formation of founder cells [7], A23187 may be acting by inducing the differentiation of amoebae into founder cells.

Though both ions appear to be involved in the A23187-mediated induction, it is not clear from our results which calcium and/or magnesium fluxes are necessary. If free intracellular calcium is estimated to be below micromolar concentrations, and intracellular magnesium to be present in millimolar quantities then one would expect a net calcium influx and magnesium efflux when the fiber is placed on agar with 1% standard salts. On agar with 1 mM manganese and 0.1 M calcium one would expect the same, namely a calcium influx and magnesium efflux, and on 1 mM manganese, 0.02 M magnesium agar a calcium influx and magnesium influx.

Generally it has been assumed that calcium has a regulatory role during slime mold aggregation. Mason et al. [8] showed that  $\text{Ca}^{2+}$  is required for aggregation in *Dictyostelium discoideum*; Chi & Francis [9] showed that substantial concentrations of cAMP caused, in a matter of minutes, an efflux of calcium, while Wick et al. [10] have presented evidence that pulses of lower concentrations of cAMP cause a rapid influx of calcium in a matter of seconds. Loomis et al. [11] have found that A23187, or the appropriate concentrations of EDTA and  $\text{Mg}^{2+}$  cause aggregation to begin sooner in *D. discoideum*. In their view, this is due to the increased accumulation of adenylyl cyclase found under these conditions. The aggregation system of *P. violaceum* differs from that of *D. discoideum*, however, in two ways: (1) In *P. violaceum* aggregation begins by the formation of founder cells, and (2) *P. violaceum* does not

use cAMP as its acrasin [12]. Furthermore, it should be noted that in no case did we find that A23187, on glass fibers or in agar, caused an acceleration of aggregation.

If a  $\text{Ca}^{2+}$  influx, assumed to be present in all the experimental conditions resulting in center induction, is responsible for the effect in fig. 1, then a stretch of the imagination is required to explain the release of the  $\text{Mn}^{2+}$  block of center induction by the addition of 0.02 M  $\text{Mg}^{2+}$ . Wasserman et al. [13] have described a similarly complex phenomenon in the case of *Xenopus laevis* oocyte maturation. They showed that it is possible to induce meiotic maturation in the oocytes by treating them with A23187 in the presence of greater than 5 mM  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ . Perhaps in both these cases  $\text{Mg}^{2+}$  acts to stimulate A23187-mediated calcium flux thru the plasma membrane, or perhaps even from intracellular stores.

In summary, we have shown that A23187 induces aggregation center formation in *P. violaceum* both at the onset of aggregation, and during the streaming process. We have also presented evidence that a  $\text{Ca}^{2+}$  and/or  $\text{Mg}^{2+}$  flux is responsible for this induction. More studies will be necessary to determine the exact physiological role of these ions during normal *P. violaceum* aggregation.

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## Cell Patterning in *Dictyostelium discoideum*

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### Introduction

In a previous review on the prestalk-prespore pattern in cellular slime molds [1], two of us (HKM and JTB) discussed how the ratio of prestalk and prespore tissue changes with aggregate size in 'slugs' of the cellular slime mold *Dictyostelium discoideum*. We presented data showing the volumes of prestalk and prespore tissue in a number of individual slugs; we also presented on the same axes data on the volumes of the stalk and spore mass from mature *Dictyostelium* fruiting bodies. The data appeared to fall on a single line; we thus suggested that the volume ratio of the two tissue types was conserved during final cell differentiation. The slope of the line (in a log-log plot with spore or prespore volume on the ordinate) appeared to be greater than 1; from this we concluded that the tissue-type ratio is shifted towards the spore pathway in aggregates (both slugs and fruiting bodies) of larger size. Skepticism about these conclusions prompted two of us (KLW and PRF) to reexamine the original data using linear regression analysis [2]. The results of this analysis are at variance with the original claims. We find that: (i) the slug and fruiting body data should not be fitted by a single line, and (ii) when lines are fitted independently to the two data sets, the lines have slopes that are less than 1. The questions which might be raised about the appropriateness of linear regression analysis (since a strictly normal distribution seems unlikely in log-log data) have been settled to the satisfaction of all authors: distribution-independent tests devised and performed by one of us (HKM) confirm the conclusions from linear regression. In this communication we describe

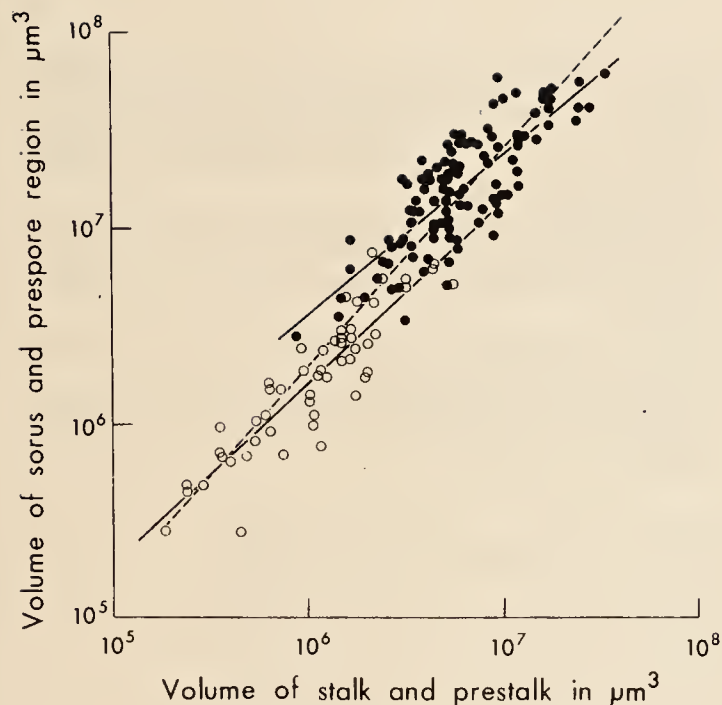
the results of the linear regression analysis and consider how the new interpretation of the data affects the ideas about patterning which were suggested in the original article.

### Linear Regression Analysis

The data published in the original review are replotted in Fig. 1; the figure shows a line fitted to the both sets of data as well as lines fitted to the data sets individually. The single line fitted to both sets of data had a slope of 1.13. However, the hypothesis that both sets of data fall on the same line was rejected with  $P$  less than 0.001. This rejection was confirmed using distribution-independent statistics; these gave a significance level of  $P = 0.02$ .

When lines were fitted to the data sets independently, they had slopes of 0.83 (for the prepattern data) and 0.95 (for the final differentiation pattern). The slope of the prepattern regression line was significantly less than 1 (probability that the slope is 1 or greater =  $2.3 \times 10^{-4}$ ), while 1 was an acceptable value for the final pattern regression ( $P = 0.64$ ). The prepattern and final pattern data could be fitted acceptably by parallel regression lines (probability that the regressions have the same slope = 0.21). The slope of the parallel lines was 0.88, significantly less than 1 ( $P = 0.01$ ). This slope indicates that *Dictyostelium* aggregates become progressively *stalkier* with increasing size; this agrees with previous results of Stenhouse and Williams [3]. Stenhouse and Williams also found an increase in the proportion of stalk in very small fruiting bodies (less than 2,000 cells). This increase could not be confirmed in the data under discussion here, because no fruiting bodies are present with less than about 4,000 cells.

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**Fig. 1.** Relationship between the volumes of prestalk and prespore tissue in migrating slugs at 20° C (filled circles) and between volumes of mature stalks and spore masses in fruiting bodies at 17° C (open circles) in *Dictyostelium discoideum* strain DD1 incubated in the dark. Data provided by JTB; these are the same as appeared in Fig. 3 of [1]. Standard linear regression analysis was used on the entire data set (dashed line) giving  $\log V_{\text{sp and psp}} = -0.48 + 1.13 \log V_{\text{st and pst}}$ . Analysis of the prestalk-prespore data alone gave  $\log V_{\text{psp}} = 1.60 + 0.83 \log V_{\text{pst}}$  (upper solid line) while the stalk-spore data gave  $\log V_{\text{sp}} = 0.52 + 0.95 \log V_{\text{st}}$ . The volumes of spores and prespores ( $V_{\text{sp and psp}}$ ), prespores ( $V_{\text{psp}}$ ), spores ( $V_{\text{sp}}$ ), stalks and prestalks ( $V_{\text{st and pst}}$ ), prestalks ( $V_{\text{pst}}$ ), and stalks ( $V_{\text{st}}$ ) are given in cubic micra

## Implications of the Reinterpreted Data

### Cell-type Conversion during Fruiting

The new interpretation of our data suggests a simplified picture of the fruiting process in slime molds. As long as we maintained that the volume ratio of the two tissue types is conserved during fruiting, we were obliged to consider that in an average-sized aggregate about 60% of prestalk cells become *spores*; this occurs since stalk cells swell (by about a factor of two) during differentiation while spores shrink twofold. According to the new interpretation in which the volume ratio shifts significantly towards stalk during culmination, cell type conversion during normal development is quantitatively minor. The data therefore suggest that *Dictyostelium discoideum* cells for the most part differentiate according to the predispositions expressed in the slug. This is in agreement with other findings [4], but in no

way implies that cell type conversion cannot occur under some circumstances.

The finding that tissue-type volumes change during culmination does not affect the validity of another point relating to tissue-type volumes of slugs that was made in the original review. The point is that when *slugs of different shapes* are compared, it is the ratio of the volumes, rather than the ratio of the lengths of the prestalk and prespore zones, which is most nearly constant. This argues against proportioning mechanisms that 'sense' tissue length, but it does not distinguish between mechanisms that "sense" tissue volumes and mechanisms that "sense" cell number or other parameters of prestalk and prespore cells in the slug. Studies on *Dictyostelium* strains at different ploidy levels indicate that the proportioning mechanism involves at least two parameters, e.g., cell surface receptors and a diffusible molecule(s) [5].

### Size Dependence of the Prestalk-Prespore Pattern

In the original review, the authors observed that an increase in the proportion of prestalk tissue in small slugs could be explained by a theoretical model developed by Gierer and Meinhardt [6]. The explanation parallels one which has been given for the increase in head proportion in small *Hydra* [7]; it depends on the idea that a pattern in the concentration of a diffusible "prestalk activator" is responsible for the "prepattern" in the slug. The allometric effect arises from the nonzero diffusion range of the morphogen, which is an increasing fraction of the slug length as the length decreases. The experimental findings reanalysed here suggest that the proportion of prestalk tissue *increases* with slug size, and hence the original explanation no longer appears to be attractive. However, these findings support those of Stenhouse and Williams [3] who examined the proportions of the mature fruiting body tissue types. They also found [3] that the proportion of stalk cells increased with *decreasing* size in small fruiting bodies as expected from the Gierer-Meinhardt model. It may be possible to modify the Gierer-Meinhardt model to accommodate the new information, but we will not attempt this here. The most significant point may be that the new analysis continues to disclose a dependence of proportions on aggregate size in *D. discoideum*. This argues against the idea that the prestalk : prespore ratio in slugs is determined by events that occur before aggregation or by purely stochastic decision-making in individual aggregated amoebae. Some sort of supracellular mechanism with



the capacity to sense slug size appears to be required. It is easy to imagine that this mechanism plays an important role in cell-type proportioning in *Dictyostelium*.

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## EVOLUTIONARY STRATEGIES AND DEVELOPMENTAL CONSTRAINTS IN THE CELLULAR SLIME MOLDS

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Over the last 30 years there has been a large accumulation of information on numerous aspects of the development and other features of the life cycle of cellular slime molds. These curious organisms arise by the aggregation of single and previously solitary amoebae to form small, multicellular structures consisting ultimately of a stalk supporting one or more spore masses or sori. Because there are relatively few known species (ca. 50) and because they are so amenable to study in the laboratory, we have acquired considerable knowledge of this microcosm of the living world. Here I would like to examine the microcosm and try to understand it in terms of what might have been the evolutionary strategies that molded the various permutations of their life histories, and how these changes might have been constrained by developmental and mechanical problems.

These questions have been asked of higher organisms and it is an obvious and well-accepted notion that a mammal, for instance, cannot indulge in just any kind of variation, but is strictly limited by the elaborate sequence of developmental steps which permit a limited number of genetic changes; all others will be lethal. In fact, the more elaborate the development, the greater are the constraints on developmental change. Furthermore, it is for this reason that most genetic variation affects changes in the later stages of development rather than in early ones. After all, a mutation in a gene affecting gastrulation has a high probability of being lethal which is not true for one affecting eye or hair color.

It is also well known and well understood in vertebrates that there is nevertheless a way of accomplishing major change. This is by heterochrony; by changing the timing of developmental events so that, for instance, some developmental processes will occur at a different time relative to others in a descendant (de Beer 1940; Bonner 1965; Gould 1977). The changes produced by heterochrony can be profound, such as larval forms producing mature eggs and sperm, or the prolongation of development to produce the extraordinary large brain in man. Such major changes are possible not only because the basic developmental plan of the organism will not be altered, but also, it is assumed, because the amount of genetic change needed to produce the alteration in timing could be relatively small. One of the important functions of regulatory genes must be to control the timing of the different elements of development.



The cellular slime molds, in contrast to vertebrates and other large organisms, have a short and very simple development. This means that the constraints are less extensive and the possibilities of making rather major changes in the development are numerous; as we shall see, some of these involve heterochrony. Another consequence of short, simple life cycles is that, for straightforward mechanical reasons, there are opportunities for convergent evolution on a scale hardly feasible in large, complex organisms. For instance, different groups of cellular slime molds whose life cycle is basically similar may have evolved independently from single-celled forms, a proposition that is obviously impossible for different orders of mammals or any of the other major groups of animals or higher plants.

Besides a desire to have a better understanding of the development and evolution of cellular slime molds, there is another purpose to this study. There is increasing interest today among biologists in the relation of development to evolution, and the question of the separation of those features of an organism that have arisen by adaptation from those imposed by a legacy of either mechanical necessity, or by a legacy of a developmental sequence of steps that cannot be ignored or bypassed. I can hardly claim that these can be clearly separated in the evolution of the cellular slime molds, but there is a considerable set of facts that bear on these questions for an interesting group of organisms.

#### THE MAJOR TYPES OF CELLULAR SLIME MOLDS

Largely through the work of K. B. Raper, L. S. Olive, J. C. Cavender, and some others we now have a good idea of the diversity of species of cellular slime molds and their relationship to one another. There are two major groups which Olive (1975) and Raper (1973) separate into the acrasids and dictyostelids. These two groups are distinguished from one another by the type of amoebae: Acrasids have amoebae with lobose pseudopodia and centrally located nucleoli, while the pseudopods of dictyostelids are filose and their nucleoli are on the edge of their nuclei. It is assumed that these are fundamental differences, and that the two groups originated from distinct kinds of solitary soil amoebae. Over and above these cytological distinctions all dictyostelids have stalk tubes made of cellulose, while acrasids lack any form of such tubes.

If we first look at the acrasids which are multicellular and have an aggregation of amoebae, we can distinguish their aggregation from that of dictyostelids by the fact that the individual amoebae never become elongate and form chains of incoming cells. Instead they remain somewhat rounded and move toward the center relatively independently. If one examines what kinds of structures are produced by the aggregates of acrasids, they fall into the following categories. (1) Some have only one cell type and the cells that are at the base of the fruiting body can reproduce and serve as spores as readily as those at the tip. In others, the stalk cells remain viable but they have a slightly different appearance from the spores. This is an extremely weak form of morphological differentiation. (2) In some species the fruiting bodies have a single knob of spores at the tip, while in others they branch to form a compound fruiting body. (See fig. 1 for a diagrammatic

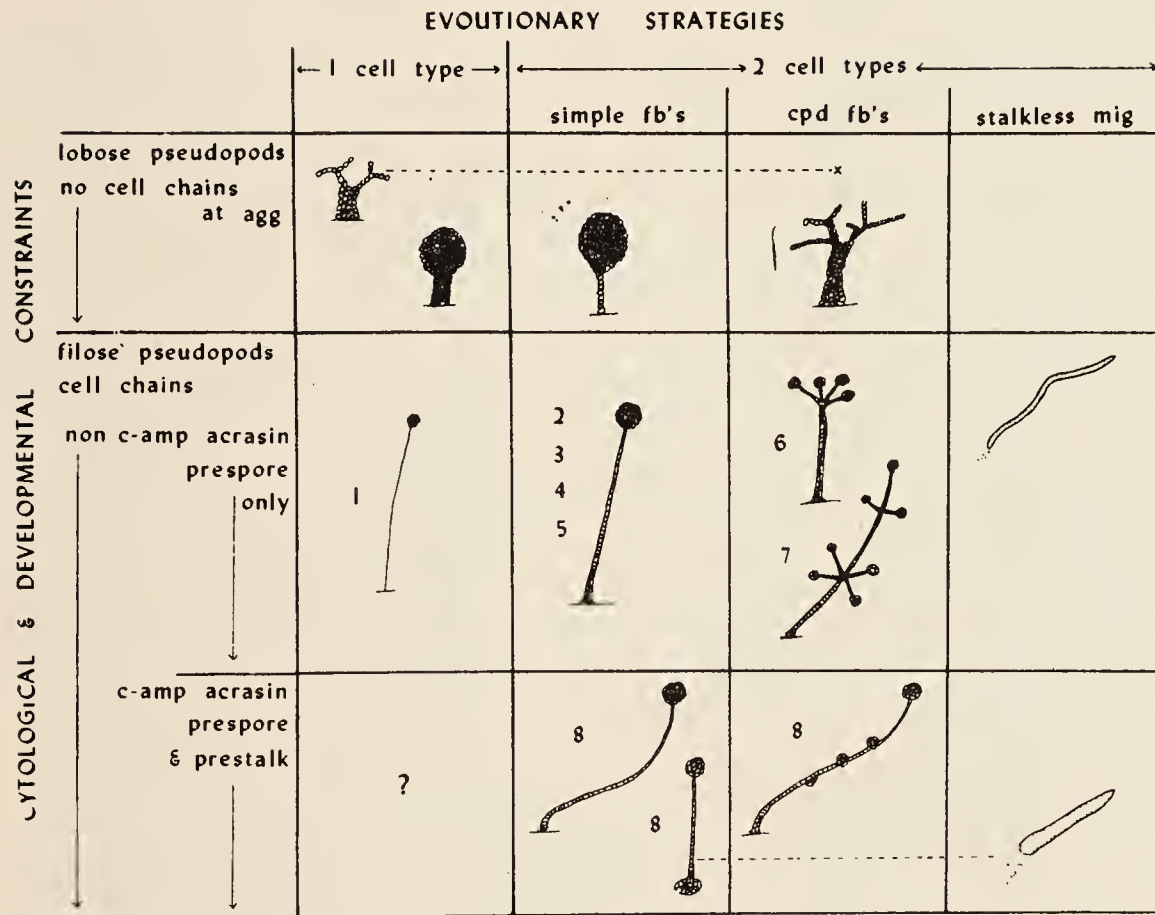


FIG. 1.—A diagrammatic chart showing three major types of cellular slime molds. Top row: the acrasids (see table 1 for names of the organisms) showing development of increasing complexity from left to right. Middle row: dictyostelids that do not use cyclic AMP for their acrasin. Bottom row: dictyostelids that have cyclic AMP as their acrasin. Numbers refer to different acrasins: 7 is *Polysphondylium* acrasin; 8 is cyclic AMP.

representation of these forms, and table 1 for the names of representative species.)

*Guttulinopsis* has a clublike fruiting body which may reach a height of 1.5 mm. All the cells have a similar crinkled appearance when mature and the stalk cells are as viable as the tip cells. The same is true of *Copromyxa*, yet it has a branched fruiting body. *Guttulina* (also called *Procheina*) and *Acrasis*, on the other hand, show primitive division of labor between the stalk cells and the spores. In *Acrasis*, the stalk cells have a ringlike thickening at the point where cells touch each other (called hilum), while in *Guttulina*, the stalk cells are smooth in contrast to the spores which appear somewhat pitted. *Guttulina* has a fruiting body with a single mass, in contrast to *Acrasis* which has a compound sorocarp and shows irregular branches. It is of particular interest that even though *Acrasis* and *Copromyxa* fruiting bodies closely resemble one another, their development is radically different (Olive 1975). In *Copromyxa*, the cells first accumulate in a mound followed by other amoebae which crawl to the tip. Ultimately, this apical accumulation of cells



TABLE I  
SPECIES NAMES FOR FIGURE 1 WITH APPROXIMATE MAXIMUM HEIGHTS (mm)

EVOLUTIONARY STRATEGIES			
	1 Cell Type	2 Cell Types	
		Simple FB's	CPD FB's
Acrasids	<i>Copromyxa</i> (3 mm) <i>Guttulinopsis</i> (1.5 mm)	<i>Guttulina</i> (0.2 mm) ( <i>Pocheina</i> )	<i>Copromyxa</i> (3 mm) <i>Acrasis</i> (1 mm)
Dictyostelids			
Acrasin not cyclic AMP	..... 1. <i>Acytostelium</i> (1.5 mm)	2. <i>D. minutum</i> (1 mm) 3. <i>D. lacteum</i> (1 mm) 4. <i>D. aureum</i> (K) (4 mm) 5. <i>D. vineo-fuscum</i> (1 mm)	6. <i>D. polycephalum</i> (1 mm) ... <i>D. polycephalum</i> (1 mm) <i>D. birfurcatum</i> (1.3 mm) <i>P. violaceum</i> (15 mm) <i>P. pallidum</i> (10 mm)
		7. [ <i>D. delicatum</i> (4.6 mm)	<i>D. mucor</i> , strains with irregular branching <i>D. rosarium</i> (10 mm)
Acrasin is cyclic AMP	..... 8. ....	[ <i>D. mucoroides</i> (20 mm) <i>D. purpureum</i> (15 mm) <i>D. aureum</i> (Car) (4 mm) <i>D. discoideum</i> (3 mm)	..... <i>D. discoideum</i> (3 mm)

CYTOLOGICAL AND DEVELOPMENTAL CONSTRAINTS

NOTE.—This list is only a small selected list of cellular slime molds to illustrate the different structures drawn in fig. 1. *D* = *Dictyostelium*; *P* = *Polysphondylium*; CPD FB's = compound fruiting bodies; MIG = migration. Note that there are two separate isolates of *D. aureum* which have different acrasins. This is expected since species are characterized by their morphology.

results in branches which are several cells thick in diameter. In *Acrasis*, there is a mass of amoebae at the tip of the stalk which, after a period of writhing movements, slowly kneads itself into a series of uniseriate, protruding branches. To anticipate a later discussion, this difference strongly suggests convergent evolution. This is supported by something L. S. Olive has pointed out to me: on the basis of other cytological considerations (e.g., the structure of the mitochondria) there is reason to believe that acrasids are polyphyletic.

Recently a wholly new and totally unrelated form has been described by Worley et al. (1979) that lends even further evidence for convergence. *Fonticula alba* has amoebae with filose filopods and produces a beautiful small fruiting body by first secreting a cone around an aggregate of amoebae, which erupt from the tip of the cone, where they ultimately produce a spherical mass of spores.

Before considering the dictyostelids, brief mention should be made of the solitary or nonsocial protostelids. These interesting organisms were discovered by Olive and Stoianovitch (see Olive 1975). There are a number of forms, for example *Protostelium*, which remain solitary, yet at sporulation each amoeba rises into the air on a delicate stalk. Protostelids appear in some ways to be closely allied to the dictyostelid *Acytostelium* (discovered by Raper and Quinlan [1958]) in which there is true aggregation including the formation of cell chains. However, each multicellular fruiting body has only one cell type. All the cells first secrete the delicate acellular stalk as they rise into the air, and then each amoeba becomes an encapsulated spore. This contrasts with most dictyostelids which have a division of labor: Some cells are part of the stalk (dying in the process) while others are lifted into the air on a rising stalk to become the sorus or spore mass.

Within the dictyostelids there seems to be a natural separation of two groups. There are (1) those forms that do not use cyclic AMP as their aggregation attractant (acrasin); in some of these the spores contain special vacuoles known as polar granules, as Traub and Hohl (1976) have shown. The other group (2) are those forms that use cyclic AMP as their acrasin; they lack the polar granules and are retained in the genus *Dictyostelium*. Another difference is that in some species in group 1 the amoebae have an alternate developmental pathway and may not aggregate; instead each separate amoeba becomes encapsulated into a spherical resistant body called a microcyst. Not all species form microcysts, but those that are known to do so in nature are all from group 1.

The majority of species of dictyostelids form simple fruiting bodies with one sorus. However, there are some species that do have compound fruiting bodies. In group 1, the two examples are *Dictyostelium polycephalum* which consists of a number of single fruiting bodies clustered together like flowers in a vase (Raper 1956) and *Polysphondylium violaceum* and *P. pallidum* which have branches that jut out in regular whorls. In group 2 *Dictyostelium rosarium* forms masses of spores from cells which periodically lag behind as the stalk rises, giving the appearance of beads on a rosary (Raper and Cavender 1968; see also Raper 1960).

One feature of the diversity that is especially pertinent to our discussion is the number of different aggregation attractants or acrasins. It was inferred from the early work of Raper and Thom (1941) and later established by Shaffer (1953) that *Dictyostelium* species and *Polysphondylium* species had different acrasins. Little



has been done since then except for some experiments of Konijn (1975) and some unpublished results of our own. All these studies are based on either putting centers of one species near the sensitive amoebae of another species or using acrasin extracts in a chemotaxis test and observing whether or not there is attraction. The only acrasin that is known chemically is cyclic AMP for various large species of *Dictyostelium*. The *Polysphondylium* acrasin has been partially purified and characterized (Wurster et al. 1976) but the chemical identity is still a matter being pursued in our laboratory.

As will be discussed later, so far we have evidence that there must be at least eight acrasins. These are numbered on figure 1; 7 is the *Polysphondylium* acrasin, while 8 is cyclic AMP. Unfortunately, our information remains very limited because only a small number of the known species have been tested; there could be many more acrasins.

With this descriptive background on the cellular slime molds, we are now in a position to examine the evolutionary and developmental issues that are the main purpose here. I will systematically look at a series of features of the development of these organisms both from the point of view of their possible adaptive strategies and their possible developmental constraints. These features are: (1) size, (2) division of labor, (3) early signs of differentiation (prestalk and prespore cells), (4) compound fruiting bodies, (5) regulation, (6) stalkless migration, (7) microcysts and macrocysts, and (8) isolating mechanisms.

*Size.*—One basic assumption in considering the adaptations of cellular slime molds is that there has been a strong selection pressure for spore dispersal. I believe that this hypothesis is sound because there is such an extraordinarily large number of species from a variety of groups of organisms that all have small, spore-bearing fruiting bodies in the range of 0.1 mm to 100 mm in height. Among the fungi there are innumerable species of small molds that fit this description; they probably can be counted in the thousands. Myxomycetes, or true slime molds, have species well into the hundreds that also fit this description. Even bacteria, in the form of some of the stalked species of Myxobacteria, show some striking examples. From this it seems to me one can only conclude that the selection pressure for fruiting bodies in small organisms, be they amoebae, hyphae, plasmodia, swarms of bacteria, or even ciliate protozoa (Olive 1978), must be enormous, and the scale of the convergent evolution vast.

Of course the dispersal strategy is not the same for all of these diverse organisms. Cellular slime molds live in the surface layer of soil and humus, dung, and dead parts of plants, and they will fruit in small cavities as well as on moist surfaces. In some cases the spores are undoubtedly spread by rain water washing through the soil. The spores of many species tend to adhere by capillarity to any object that touches them, and therefore it is presumed that they depend on small soil invertebrates brushing past them and spreading the spores. In a few species, such as *Acrasis* the spores are dispersed by the wind. This raises the question of what environmental factors might govern the size of the fruiting bodies.

The upper end of the size range for various species of cellular slime molds varies from roughly 0.2 mm to 20 mm. This is the height of the fruiting bodies and does not necessarily reflect differences in the thickness of the stalk or the quantity of

the spores. It is more difficult to estimate the mean size because this depends so much upon the amount of food present. Also it must be remembered that all our measurements come from amoebae grown in the laboratory; we know nothing of the size ranges in nature. It is important to recall that the size of a fruiting body is not directly related to growth, as it is in most multicellular organisms. Here growth occurs first by multiplication of the separate amoebae, and after the period of growth and feeding the cells aggregate. So the size of a particular aggregate, at least at the lower end of the scale, depends upon the mass of the amoebae that joined one center, and the abundance of food will only have an indirect effect on size.

Unfortunately we do not know any details concerning their habitats and can only speculate why some species are larger or smaller than others. For instance, L. W. Buss has pointed out to me that it might be an adaptation for dispersal by different sized or shaped soil animals, an intriguing idea that he is in the process of testing. Since various species will co-exist in the same patch of soil we do know that they must have separate niches in the same habitat, but we have no clue as to whether these niches include different modes of dispersal and whether each of them has a size optimum. From the work of Horn (1971) it is clear that food preferences play a crucial role in coexistence; different species and strains of cellular slime molds will selectively feed on different soil bacteria in any one area.

Some time ago it was suggested to me by Robert MacArthur that smaller fruiting bodies might have an advantage by developing more rapidly, in this way quickly becoming spores before a rapidly changing environment becomes lethal. Unfortunately this turns out to be true only for the small fruiting bodies of any one species (Bonner and Eldredge 1945). If one compares the average time taken from fruiting of a small species such as *D. minutum*, with a large one such as *D. mucoroides*, they are comparable; the small size of *D. minutum* gives it no advantage in speed of development. If a particular size of species is adaptive, the reasons must be sought elsewhere. It should be noted that in this respect slime molds differ radically from all other organisms in which growth occurs concurrently with differentiation. In the latter, the larger the organism, the longer it takes to reach maturity: A bacterium can produce a generation in a fraction of an hour, while a generation in a giant sequoia takes 60 yr (Bonner 1965, p. 17).

If size is under selective forces, then it is reasonable to suggest that height may be only of partial significance; the total mass of spores is of equal or perhaps even greater importance. It is certainly true that species will differ in their proportions. If this is the case, why are not all species very large with many spores? We certainly do not understand the adaptive aspect of the problem, although there may be something to say on the side of developmental constraints.

It has been known from early observations that it is impossible, by having a very heavy culture of amoebae, to produce giant fruiting bodies. There always seems to be an upper limit. A large aggregate will break up into a group of cell masses, each crowned with a tip, and each will produce a large fruiting body. Hohl and Raper (1964) did this artificially by putting cells in a roller tube and showing that large groups of adhering cells subdivide into smaller units of consistent size.



More recently, in our laboratory, Kopachik (1980) has shown that this process involves the formation of dominant tips which divide the large cell mass according to their strength and the sensitivity to domination of the surrounding cells.\*

This is a first step in our understanding of how an upper size limit can be governed, but here I am asking why is there such a limit. One answer is that there is a mechanical size limit above which amoebae cannot construct a stalked fruiting body. The argument in favor of such mechanical constraint is that fungi, which are constructed with filaments, can and do manage very large fruiting bodies, the most notable of which are mushrooms. Of course there are small molds too, so with both fungi and cellular slime molds I would like to suggest the following hypothesis: A range of smaller sizes of fruiting bodies is adaptive, while the upper size limit is constrained by mechanical considerations (as well as the accessibility to oxygen) set by the limitations of amoebal building bodies. In other words, any very large mass of cells in the cellular slime molds would be unable to develop if it tried to produce one giant fruiting body. Size selection might also be involved in conjunction with these mechanical problems. Collapsed fruiting bodies will be selectively disadvantageous, so any gene or set of genes which impose a size restriction will be under positive selection.

The idea that a size limit would be produced by a developmental constraint is a very general principle and applies to all organisms. In the case of slime molds let us consider briefly possible ways in which these upper size limits might be imposed; what are the possible mechanical considerations? They could, for instance, be limited by their mode of development. As Kopachik (1980) has pointed out (following the theoretical considerations of Crick [1970]), they could be limited by the distance necessary for the diffusion of key substances (e.g., morphogens) during development.

Another possibility concerns the limitations imposed by gravity. Slime molds with cellular stalks do achieve a greater height than *Acytostelium*, although as can be seen from table 1, the difference is not striking and many are shorter. However, the size of sorus or spore load is greater in species with cellular stalks; the whole fruiting body is of a sturdier construction and can hold a larger mass of spores (fig. 1). One presumes the reason for this is that the dead cells in the stalk form cross walls of cellulose before they die (resembling the pith of a higher plant) and that these cross walls serve as struts. A tapering cellular stalk certainly gives the appearance of a more robust structure than the delicate acellular stalk of *Acytostelium*.

Whether or not this apparent increase in strength is significant is a more difficult matter to ascertain. As McMahon (1973) has shown, large trees have a relation between their height and their diameter of height  $\propto$  diameter<sup>2/3</sup>. He points out that this relation is necessary in order for the tree not to buckle under its own weight; the larger trees must become disproportionately thick, and the above relation of height to thickness of the tree can be predicted if it is assumed that elasticity is the limiting factor (McMahon's "elastic similarity model"). The fruiting bodies of *Dictyostelium discoideum* resemble trees in that they also have tapering trunks. If one measures their height and their diameters at the midpoint of the stalk, then the

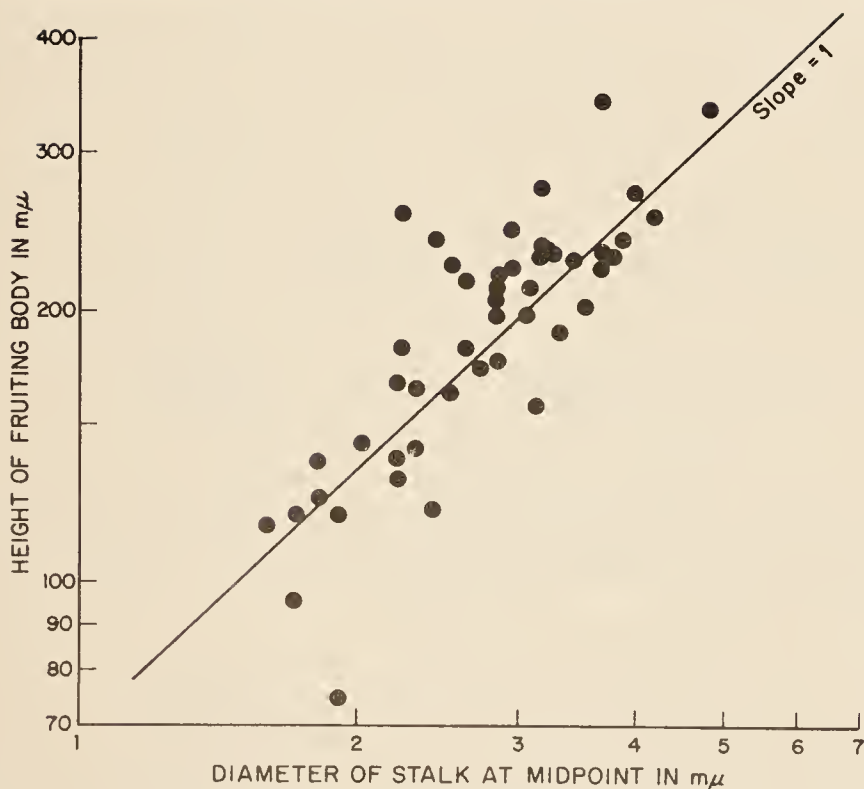


FIG. 2.—A log-log graph of the height of a fruiting body of *D. discoideum* and the diameter of its stalk at midpoint. Note that the slope is approximately 1, which is expected if the different sized fruiting bodies are geometrically similar.

relation is different than trees; height varies directly (or very close to it) with diameter (fig. 2). In other words, *D. discoideum* fruiting bodies are geometrically similar; if their height is doubled, the diameter of the stalk will double.

One assumes that the reason for this difference between trees and slime molds is gravity. The weight of trees is the factor which would cause buckling, but the weight of minute cellular slime molds is negligible and as Rorke and Rosenthal (1959) showed the effect of gravity on slime molds is minimal. There is no mechanical need to become disproportionately thick with increasing size. Therefore small size eliminates the mechanical constraints imposed by gravity, and the result is simple geometric similarity of different-sized fruiting bodies. But if the fruiting bodies were to become any larger, in order to stand they would have to evolve in proportions consonant with the relation required by elastic similarity, a step which might involve a major set of genetic and accompanying developmental changes. Perhaps millions of years from now this hurdle will be surmounted and the world will become populated with giant slime molds.

*Division of labor.*—Some dictyostelid cellular slime molds have an acellular stalk (*Acytostelium*) while all others have a true division of labor with some cells becoming stalk cells and the others spores (fig. 1). The presumed disadvantages of the method of fruiting body formation of *Acytostelium* is that each cell must expend considerable energy secreting a cellulose stalk at the cost of the energy



reserves for the spores. Those slime molds that do divide the labor can permit the spores to keep virtually all their stored energy except that necessary to secrete the cellulose spore capsule, while the stalk cells secrete all the cellulose of the stalk and then become trapped in the cellulose cylinder they deposit. However, there is an enormous sacrifice of cells: Depending upon the conditions and the species, anywhere from 20% to 90% or more of the cells die in the process of fruiting. As I have pointed out elsewhere (Bonner 1980) this may be considered a striking case of altruism for the stalk cells die as they become incorporated into the stalk (Whittingham and Raper 1960). The stalk cells and spores may be closely related genetically, perhaps often identical, which means that this altruism could be helped by kin selection. We must assume that the advantages of producing a fruiting body, and therefore achieving more effective dispersal, must outweigh the disadvantages of losing a large number of the cells during development. The fact that all the cells are related, if not genetically identical, makes it easy to see that from the point of view of gene preservation, the sacrifice is probably not so great as originally imagined.

Next comes the question of the proportion of stalk cells and spores. Many workers have made measurements; let me give the recent figures of Takeuchi et al. (1977). They estimate that in *D. discoideum* 21% of the cells are stalk and basal disk, 68% spores, and 11% of the cells are undifferentiated. Previous workers had ignored the undifferentiated cells, but that these are often present inside the mature sorus has been frequently observed in our histological preparations. It is a moot question whether they are the result of suboptimal fruiting conditions, or the result of some developmental error or untidiness, or whether they serve some function, such as helping in the raising of the sorus up the stalk.

In those forms which continuously form stalk as they migrate, for example, *Dictyostelium mucoroides* or *Polysphondylium pallidum*, the percentages of spores and stalk cells will be extremely variable depending on how much they migrate. If they fruit immediately the percentage of spores will be high and the longer they migrate, the smaller the percentage. In some extreme cases, where the optimal conditions for migration are carefully maintained, the final sorus may be minute, and the stalk enormously long (up to 22 cm; Bonner and Shaw 1957).

From this one might conclude that there is great selection pressure to fruit in the right place, and hence the long migration. In those species that make a stalk as they migrate, the enormous cost of losing over 90% of their cells must be outweighed by the advantage of getting a few spores to the right place for favorable germination and growth. The constraint is the large loss of cells in the long stalk; in a later section I will consider the question of stalkless migration. It is conceivable that the long stalk is useful in allowing the cell mass to bridge chasms or gaps in the soil; a stalked migrating slug can go some places that would be impossible for a stalkless slug.

*Prestalk and prespore cells.*—It has been known since the work of Bonner et al. (1955) that certain species of cellular slime molds, such as *D. discoideum* and *D. mucoroides*, show early signs of differentiation in the migration stage. The pre-stalk and the prespore cells have different staining properties and numerous characteristics revealed by a number of workers since then (review, MacWilliams

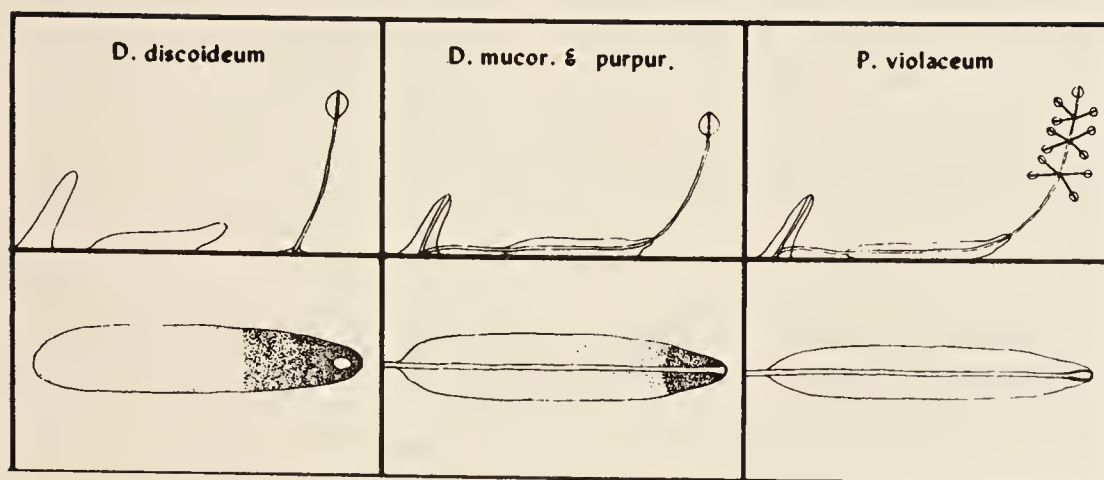


FIG. 3.—Diagrams comparing various dictyostelids with respect to their morphology during development (top row) and their PAS staining reaction which shows the presence or absence of prestalk (dark stipple) and prespore (light stipple) cells (bottom row). (From Bonner et al. 1955).

and Bonner 1970; my fig. 3). Other species, such as both species of *Polysphondylium* and *D. polycephalum*, show no such early signs (Bonner et al. 1955). However, recent electron microscope work has revealed that in the migration stage there is a uniform presence of prespore or PV vacuoles which are characteristic of prespore cells (Hohl et al. 1977). It would be more correct to say that some species have only prespore cells, while others have both prespore and prestalk cells.

It is of particular interest that those species that possess both presumptive cell types in distinct zones are the ones that use cyclic AMP for their acrasin (table 1). At least, in all cases tested the correlation has been perfect. This raises many questions: for instance, does the cyclic AMP-acrasin mechanism impose a constraint on the development that automatically produces prespore and prestalk zones? Or is the correlation mere coincidence? This is a matter of great interest. Cyclic AMP is apparently involved in the differentiation process of all species tested (George 1977; Hohl et al. 1977), but some also use cyclic AMP as an acrasin and they alone show the early signs of differentiation in clearly defined regions. Recently Schaap et al. (1981) have shown that in *D. minutum*, which is not a cyclic AMP acrasin species, there are prestalk and prespore cells. But they are irregularly distributed; the correlation only works when they are in distinct anterior and posterior areas.

Just as the final ratios of stalk cells to spores show consistent proportions with different size fruiting bodies, so do the prespore and prestalk areas of the slug (Bonner 1957; MacWilliams and Bonner 1979). There is some evidence that the proportion is controlled by the volume of the prestalk and prespore zones, but the extraordinarily interesting problem of the mechanism of this control remains totally speculative.

Finally, let us return to the question of why some species show the presumptive zone and others not. Some years ago we suggested it might be a matter of timing:



that differentiation proceeds in a series of steps and that these steps can either progress slowly and be spaced out, as in the larger species of *Dictyostelium*, or can all occur quickly at the end, as in *Polysphondylium* (Bonner et al. 1955). As we pointed out, by delaying these early steps of differentiation, *Polysphondylium* may be considered neotenuous, that is, there has been a prolongation of the youthful period of development. In this way it is possible for *Polysphondylium* to quickly generate new, minute fruiting bodies which become the branches. If there had been presumptive stalk and spore zones, the time for regulation might have made such a pattern difficult. A possible fallacy in this argument is that some strains of *D. mucoroides* will often form irregular branching. However, the fact that they are irregular suggests that they come from groups of cells that already are committed to some degree to form small fruiting bodies, and therefore do not divide the space around the parent stalk in the perfect whorls found in *Polysphondylium*. We will return to this matter when we consider compound fruiting bodies. Here let me conclude by pointing out that genes that affect timing and shift the moment at which certain developmental events occur (heterochrony) can have profound effects on the final outcome of development. As pointed out in the beginning of this paper, this is an old story for many higher forms, but the principle of evolutionary change by heterochrony may equally apply to the cellular slime molds.

*Compound fruiting bodies.*—As can be seen at a glance from figure 1, the variety of compound fruiting bodies is considerable. Furthermore, the evidence for convergence is strong. It has already been pointed out that *Copromyxa* and *Acrasis* produce much the same shape, yet their mode of development and branch formation is totally different. The same point can be made contrasting the compound fruiting body of *Dictyostelium polycephalum*, *Polysphondylium*, and *D. rosarium*.

In each of the latter species it is possible to interpret their differences in terms of heterochrony. As before, this is highly speculative. Let us begin with the differences between *Polysphondylium* and *D. rosarium* because we have already made the argument that the former might make complete branches with stalk cells because it has delayed the early stages of differentiation until the last moment. *Dictyostelium rosarium*, on the other hand, is a cyclic AMP species and does have prestalk and prespore zones, as A. Hamilton has shown recently in our laboratory. Nevertheless it cuts off groups of cells along the stalk as it rises into the air in a manner identical to that of *Polysphondylium* (fig. 1). The buds of *D. rosarium* do not turn into small fruiting body branches; they transform directly into a sphere of spores. Apparently they have already followed along their course of differentiation sufficiently far that they do not regulate and form new stalk cells. Heterochrony would seem to explain satisfactorily the difference in form between these two species.

The case of *D. polycephalum* is somewhat different in detail. It is a noncyclic AMP acrasin species and the migrating cell mass or slug has no prespore and prestalk zones (Bonner et al. 1955). At the end of migration the slug breaks up into a cluster of small single fruiting bodies which rise together into the air (fig. 1). We may presume that the reason the migrating slug can subdivide in such a way before

final fruiting is that it is totally undifferentiated, and does not have to reverse itself in any way. This implies that there could not as easily be such a compound fruiting body in a cyclic AMP acrasin species because they show partial differentiation and would have to undergo dedifferentiation. Again the basic postulate is that the timing of the differentiation process has permitted different fruiting body patterns.

There is one curious form of branching, or more properly a substitute for branching, found in *D. mucoroides* var. *stoloniferum* and described by Cavender and Raper (1968). This strain, which comes from the rain forests of Costa Rica, has a long stalk which seems to elongate near the surface of the substratum and when it forms terminal spores, they soon touch and spread onto the substratum. As we showed many years ago and has been much studied since then, *D. mucoroides* and all other species tested have an autoinhibition of germination, but perhaps this variety *stoloniferum* lacks the inhibitor (or a sensitivity to it) and all the amoebae from the collapsed sorus germinate. If no food is present, they aggregate in a matter of a few hours and form a smaller secondary fruiting body which rises at the new site. This remarkable strain of Cavender and Raper not only has the equivalent of the advantage of branching, but if there happens to be food present where the first sorus falls, it can switch to the vegetative stage and produce an even larger fruiting body as a result of growth. So this method of substituting for branching, simply by omitting the secretion of the germination inhibitor (or being insensitive to it), is facultative.

*Regulation.*—One of the most outstanding features of the multicellular forms of the cellular slime molds is their ability to regulate. Many years ago Raper (1940) showed that if a migrating slug of *Dictyostelium discoideum* was cut into fractions, each cut portion developed into a complete fruiting body with spores and stalk cells, even though the cell fraction came from cells that originally would have produced one cell type only. Much work has been done since then and we know many details of the phenomenon. In *D. discoideum*, where there are prestalk and prespore cells, it is clear that prestalk cells can be converted to prespore cells, and vice versa. In this case determination is preceded by the first steps toward differentiation. The process of regulation in noncyclic AMP species that lack the early signs of differentiation is even more obvious; in fact, in *Polysphondylium* what amounts to regulation occurs each time a branch is formed.

The pertinent question here is whether this property of regulation has arisen by natural selection in the cellular slime molds. A good case could be made for regulation being adaptive because it is obvious that an accident to the migrating cell mass will not cause its loss, but the fragments can re-form to fruit. It provides the same advantage for slime molds as regeneration by growth provides in higher animals and plants. Therefore, if we assume that branching in some ecological circumstances can be advantageous for more effective dispersal, then obviously regulation will be advantageous, since it is used as the basic mechanism of branching.

However, it is impossible to answer the original question of whether regulation arose by selection or whether it was a property that appeared for other reasons. An argument could be made for the idea that it is a gift which was automatically provided because of the mechanisms of development and its possible adaptiveness



is therefore a sort of bonus. It is hard to imagine that the alternative, namely a mosaic development, is possible in an aggregative organism. While there is evidence that some of the prespore and prestalk characters are present before aggregation and that they subsequently sort out (Tasaka and Takeuchi 1981), this is hardly the same thing as having determined cells before aggregation. It would be very difficult to achieve constant spore-stalk ratios were this the case because to do so each aggregation field would have to have the correct ratio of determined, presumptive spore and stalk cells. Therefore, the fact that differentiation remains reversible until mature spore and stalk is formed is an essential part of the mechanism of development. Since both reasons for the existence of regulation in the cellular slime molds seem reasonable, perhaps the best way to put it is that regulation is adaptive because it permits a mechanism of development that leads to controlled spore-stalk ratios, which is presumed to be under positive selection because of effective spore dispersal.

*Stalkless migration.*—There are four known species with stalkless migration: *Dictyostelium polycephalum*, *D. dimigratoform* (Cavender 1970), *D. intermedium* (Cavender 1976), and *D. discoideum* (fig. 1). Previously I stressed the possible advantage of migration to dispersal. Among other things it brings the spore-bearing structure away from the region of feeding to regions which might be quite different. The migrating slug of *D. polycephalum* does not seem to orient to light (Raper 1956), but it is well known that the migrating slug of *D. discoideum* is positively phototactic and thermotactic (Raper 1940; Bonner et al. 1950; Francis 1964; Poff and Butler 1974; Poff and Skokut 1977). More recently Whitaker and Poff (1980) have shown that the thermotaxis becomes negative in a lower temperature range. One can imagine, for instance, that in the warm daytime migration will be toward the warmer surface, but in the cool of the night it will be toward the colder soil surface. This is pure conjecture and it would be of considerable interest to measure the actual gradients in the soil under different conditions.

The great advantage of the stalkless condition must be that extended movement can occur without the severe loss of stalk cells. That there is some cell loss and that the slugs of *D. discoideum* get smaller as they migrate is clear (Bonner et al. 1950) but the amount of loss is less than in migration with stalk formation.

Again stalklessness may be considered in terms of developmental timing. In a form with a stalk during migration the stalk begins forming as soon as the first papilla arises in the cell mass after aggregation. In a stalkless form the onset of stalk formation is delayed until after the period of migration. Raper and Thom (1941) showed many years ago that some strains of *D. mucoroides* and *D. purpureum* have periods of stalkless migration and it is easy to imagine that the true stalkless condition arose through the further modification of such forms. In fact, *D. dimigratoform* and *D. intermedium* mentioned above do show somewhat intermediate characteristics. Recently, Takuzo Yamada has sent me photographs showing *D. discoideum* with a stalked migration and no basal disc, making it appear very much like *D. mucoroides*. He achieves this phenocopy by treating the spores with urea. Yanagisawa et al. (1967) produced a mutant which shows a similar shift in morphology. It would be of great interest if one could learn more about the timing of stalk formation and how it is genetically controlled.

*Macrocyts and microcyts.*—Macrocyts are the sexual stage, the zygote of the cellular slime molds (Blaskovics and Raper 1957; Erdos et al. 1972, 1973; Filosa and Dengler 1972). They are known to be present in many different species of dictyostelids, but so far they have not been demonstrated in acrasids. Some strains appear to be homothallic and others heterothallic (Clark et al. 1973; Erdos, et al. 1973). In the homothallic forms the recombination advantage of sexuality will only occur when an aggregate is composed of cells of different genotypes. If their advantage in sexuality is circumscribed, could they have other adaptive features? It is well known that the formation of macrocyts is favored by excess water; they appear under submerged conditions. This is true of all types of known macrocyts; other environmental conditions such as the effect of light and carbon dioxide have different effects on hetero- and homothallic forms (Weinkauff and Filosa 1965; Nickerson and Raper 1973; Erdos et al. 1976; Filosa 1979). From this information one might make the hypothesis that macrocyts serve two functions: For some species the role of sexual recombination is especially important, while for all species it is a resistant stage for flooding conditions. To illustrate the point, I cite the work of Filosa et al. (1975) who showed that in a homothallic strain of *D. mucoroides* one can convert migrating slugs to macrocyts by adding a deep layer of water. However, heterothallic forms, which are also stimulated to form macrocyts in deep water, cannot change their course of development once the macrocyst development process has been initiated at an early pre-aggregation stage (Wallace 1977; D. Bozzone, personal communication). It is almost as though in homothallic forms, the adaptation to flooded conditions is perfect and can be shifted at short notice in the event of a downpour of rain, while in heterothallic forms the ability to shift is severely constrained, but this disadvantage is compensated for by the advantage of greater sexual recombination.

Microcyts have only been described so far in noncyclic AMP acrasin dictyostelids. Individual amoebae, before aggregation, become encysted in a resistant case. It is presumed that this is an alternative pathway of development, an emergency encystment if conditions do not favor aggregation and normal fruiting. In an interesting study Francis (1979) gave evidence that different proteins are synthesized for the microcyst pathway from those found in normal development, and Hohl (1976) demonstrated that the structure of the spore capsule differs significantly from that of true spores of the same species. It was shown by Toama and Raper (1967) that putting the amoebae of *P. pallidum* in a high concentration of certain ions induced the formation of microcyts. A more likely natural inducing agent for amoebae in the soil was discovered by Lonski (1976) who found that small amounts of ammonia were exceedingly effective in inhibiting fruiting bodies and stimulating microcyst formation. This would seem to imply that if the soil was rich in bacterial activity the microcyst pathway would be favored. It is not clear, however, if this is a genuine adaptive strategy.

*Different acrasins.*—One of the most intriguing problems for the future is the question of the origin of different acrasins for different species. As can be seen from figure 1 and table 1, eight are already suspected and no doubt with further study more will be found.

It is not difficult to imagine why there are so many acrasins, for as Raper and



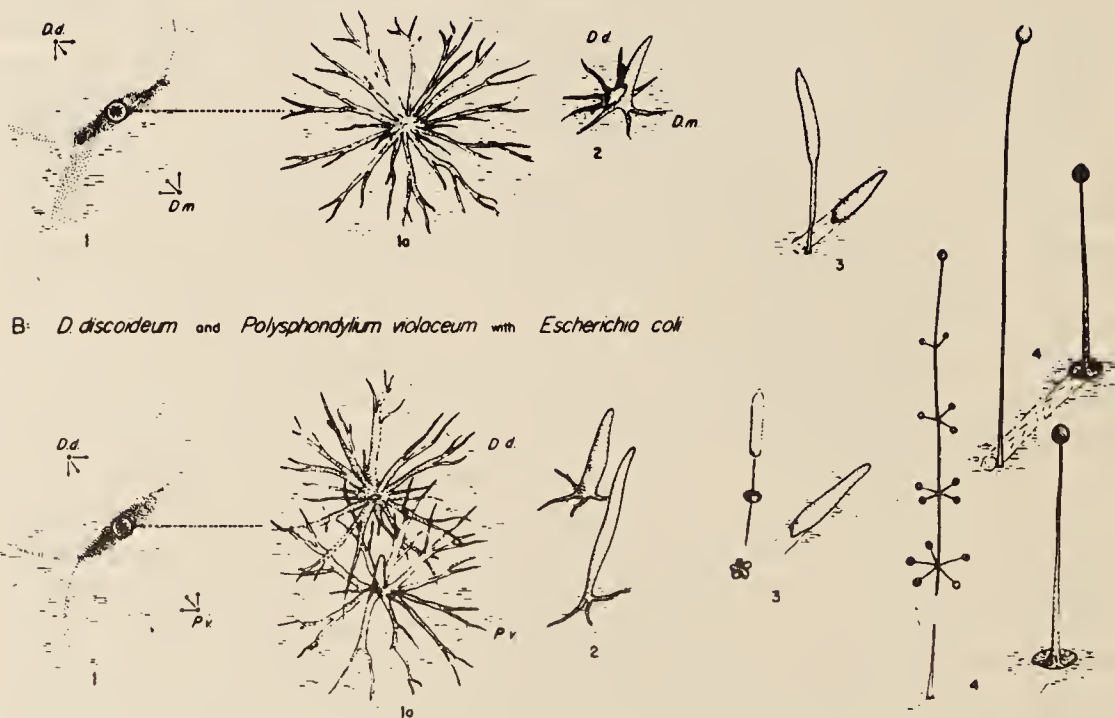
A *Dictyostelium discoideum* and *D. mucoroides* with *Serratia marcescens*

FIG. 4.—A, Two species which co-aggregate but whose tips subsequently separate at the center of the aggregate. (The pigment from *S. marcescens* only is retained by *D. discoideum* and therefore the cells of the two species can be identified). B, Two species which have separate acrasins and aggregate with overlapping streams to their individual centers. 1, aggregation in a region containing amoebae of both species; 2, the end of aggregation showing the newly formed tips of both species; 3, migration stage; 4, final fruiting bodies. (From Raper and Thom 1941).

Thom (1941) showed long ago, they serve as effective isolating mechanisms between species. If diversity and speciation are to occur in the slime molds, there will be a high premium for effective isolating mechanisms as evolution proceeds. Two well-established mechanisms are easy to demonstrate. One is in closely related species, such as *Dictyostelium mucoroides* and *D. purpureum* (purple sorus; Raper and Thom 1941; my fig. 4). This separation apparently has to do with differences in the adhesive properties of the cell surfaces, and as has been shown in three studies, the separation can be accounted for by Steinberg's (1970) differential adhesion hypothesis (Nicol and Garrod 1978; Sternfeld 1979; Gerisch et al. 1980). Differences in ability of cells to remain together also can be shown in different strains of the same species (Bonner and Adams 1958). The other isolating mechanism is having different acrasins. Raper and Thom (1941) showed elegantly that if *P. violaceum* and *D. discoideum* are mixed, and one species vitally stained, the streams of one species will ignore and overlap the other; they are responding to totally different signals (fig. 4).

Let us now turn to the more difficult question of how these different acrasins arose during the course of evolution. The best way to examine the problem is to look at a cyclic AMP acrasin species to understand what an acrasin system

involves in the way of complexity (for a review see Newell 1977). First an enzyme is necessary to synthesize the attractant (adenyl cyclase) and to inactivate it (phosphodiesterase). A membrane mechanism is necessary to allow the cyclic AMP out of the cell when the cell is stimulated by external cyclic AMP. This process involves a receptor protein on the surface which specifically binds with cyclic AMP. How the membrane then becomes more permeable to cyclic AMP is not understood, although there is evidence that calcium ions may be involved. There is also a cyclic AMP binding protein inside the cell which in turn may be responsible for the oriented movement of the cell, although this is pure conjecture because our understanding of the mechanism of amoeboid movement and its orientation by cyclic AMP is obscure.

Only one other attractant is known chemically and that is folic acid (Pan et al. 1972). There is no evidence for its being an acrasin and in some species, including *D. discoideum*, it is more effective on vegetative than aggregating amoebae. For this reason it has been suggested that it is used by the vegetative amoebae to find food, for bacteria secrete folic acid. However it is known that folic acid can act synergistically with cyclic AMP (Bonner et al. 1970; Wurster and Schinbiger 1977). Since we do not know and have not even looked at the acrasins of many species of cellular slime molds there remains the possibility that folic acid serves as an acrasin for one or more species. Certainly some of the species with unknown acrasins respond strongly to folic acid (unpubl. results). It is also established that there is an enzyme produced by the amoebae which inactivates the pteridine ring of folic acid (Pan and Wurster 1978), but nothing is known of the receptors and the other associated molecules.

For some time we have been working on the identification of the acrasin of *Polysphondylium violaceum* (Wurster et al. 1976). It is clearly neither cyclic AMP nor folic acid. Like cyclic AMP it is a small molecule, which is secreted in large quantities just at the moment of aggregation, and there is an enzyme which inactivates it. With the expert help of O. Shimomura we now have methods of preparing small quantities of the material in a relatively pure state and hopefully we will eventually know its structure. It is also a strong attractant for *P. pallidum*, but we do not know if *P. pallidum*'s acrasin is identical or merely closely related. *Polysphondylium violaceum* acrasin also strongly attracts *D. bifurcatum* and *D. delicatum* (unpubl. results). The other evidence for diversity in acrasin comes from Konijn (1975) who mixed *D. lacteum*, *D. aureum*, *D. vineo-fuscum*, and *D. polycephaleum* in pairs in different concentration ratios (1:10, 1:1, 10:1) and found no coaggregation or mutual attraction. (The above is the basis for acrasins 3,4,5, and 6, indicated on fig. 1).

This brings us back to the crucial question, How did these different acrasin systems evolve? Perhaps we shall be in a much better position to answer this question when we know more of the chemistry of these substances, but that information may be slow in coming. At the moment we can only speculate, and here I shall consider the relative merits of two hypotheses, which are not, as will be obvious, mutually exclusive. They are: (1) that there is an independent origin from single-cell soil amoebae of each group of species with a unique acrasin, and (2) a species with one acrasin system gave rise to another.



1. The basic argument in favor of an independent origin is that the acrasin system is so complex that it might be prohibitively difficult to evolve from one acrasin to another. A set of about a dozen interrelated substances are needed and these must be built up so that their complex relation to one another is in perfect balance. The idea of altering one acrasin system into another seems hopelessly involved, and one alternative is to start anew from single cells. No doubt there has been plenty of geological time although unfortunately there is no cellular slime mold paleontology.

I have already stressed that there is a powerful selection pressure for small fruiting bodies producing massive convergence among slime organisms. Here the argument would be that the convergence is massive and each acrasin aggregation system arose independently from ancestral solitary soil amoebae.

If one looks to morphology, there is only weak supporting evidence. We know nothing of the acrasins of the acrasids, but clearly they are of a radically different stock than dictyostelids, and it is easy to imagine that the two groups arose independently (fig. 1, table 1). It is presumed that the basic differences in the construction of the amoebae of these two major groups fully justifies this conclusion. Among the dictyostelids there is a separation into two major categories which we have called the cyclic AMP acrasin forms and the noncyclic AMP forms. The cytological differences between these two groups are few, but there is one that has been emphasized by Traub and Hohl (1976). They showed that the spores in some of the noncyclic AMP acrasin species have polar granules which are totally absent in the cyclic AMP acrasin forms. Whether or not this correlation is perfect is not yet certain, but it does again suggest that at least these two major groups of dictyostelids could have had an independent origin from the unicellular state. If we now look at the noncyclic AMP forms, some of which are very similar except for their acrasins, the argument for independent origins becomes more difficult. So much so that one would wish to examine the second, alternative hypothesis closely.

2. We are now confronted with the apparently difficult problem of converting one acrasin system, with its complex of specific, interrelated substances, into another. At first this seems almost impossible to imagine, but two suggestions can be made. One is that all the acrasin system genes are closely linked on a chromosome, and that there has been a transfer of a segment of DNA containing this entire genetic material from one form to another. It is now known that such transfers are entirely possible, but there is a problem. Where will this gene package come from? If it has already evolved independently as an acrasin system, it seems rather pointless to transfer it to another species; this hardly represents evolutionary progress.

A second possibility seems much more appealing, although because of our ignorance it is tantalizing. The two chemoattractant systems that we know use two substances that are common to all amoebae, cyclic AMP and folic acid. For totally different metabolic reasons these substances will have all the major elements of an acrasin system encoded in the genome, and present in the cell. All that is needed to make them a unique acrasin is to see that their receptors predominate on the cell surface at a key moment in development. If this hypothesis is correct, then an

acrasin could be any small (and therefore readily diffusible) molecule normally synthesized and degraded in the cell, and with receptor and other associated proteins already present. That there might be eight or more such common metabolites in amoebae is certainly reasonable. As a corollary it would also be reasonable to find that some species of cellular slime molds do use folic acid as their acrasin. It is indeed frustrating that we do not know such is the case, nor do we know the chemical structure of *P. violaceum* acrasin or the acrasin of any other species.

Again, if there is anything to this hypothesis, note that it brings us back to a previous conclusion. In order to elevate a low molecular weight metabolite to the status of an acrasin two things are needed: one is the increase in synthesis of cell parts of the new acrasin system with the corresponding decrease of the ancestral acrasin. The other is that the acrasin must increase at a key moment in development, a certain period of time after starvation when fruiting should be optimally advantageous. So we are back to the question of genes which control timing; they must be an integral part of the efficacy of the acrasin system.

Finally, as K. Inouye has pointed out to me, the problem here is not unlike the diversity of neurotransmitter substances found in animals where there also has been an evolution and proliferation of chemical systems involved in cell to cell communication. In both cases, acrasins and neurotransmitters, the effects on the cells are similar, yet the chemicals involved vary widely. The only property they seem to hold in common is that they are relatively small, stable molecules.

#### SUMMARY AND CONCLUSIONS

I have tried to show where, in our present state of knowledge, the evolution and the development of the cellular slime molds meet. Numerous aspects of their development have been examined to see whether or not they would best be considered adaptations or constraints, either mechanical or developmental. The answer to each question is admittedly highly speculative, but together they give insights into these curious organisms that may be helpful to future research on their development and their evolution.

1. It is reasonable to postulate that their size is affected by selection. This would be considered to include their evolution into multicellular organisms (i.e., aggregation) and the control of their minimum, but possibly not their maximum size which may be limited by such mechanical constraints as their amoeba building blocks, size limits for the diffusion of morphogens, and oxygen for metabolism.

2. The labor is divided into stalk and spore cells, but the reasons that this is so are harder to assign. It could be adaptively advantageous to conserve the maximum energy in the spores, or it could be a mechanical result of size as it is in all large animals and plants.

3. The best guess for the presence of early signs of differentiation in the form of prespore and prestalk regions is that it is a constraint imposed on those species that use cyclic AMP for their acrasin. This is a matter that perhaps can be settled by further investigation, beginning with learning the chemical nature of other acrasins.



4. Branched fruiting bodies may well be more effective in spore dispersal under certain environmental conditions and, therefore, adaptive. The form of the branches, however, are probably constrained in significant ways by the mode of development.

5. It was concluded that regulation in fragmented portions of a cell mass could either have arisen by selection, or could be an inborn property of the aggregation method of becoming multicellular. Therefore, regulation might be both adaptive and a developmental constraint at the same time.

6. Migration and the presence or absence of stalk during migration are most easily explained by postulating selection favoring mechanisms to reach a better place to put the spores for dispersal. It is possible that stalked migration is a legacy from small ancestors, yet there is also the possibility that long stalks are adaptive and can put the spores in optimal positions that cannot be reached by stalkless migration.

7. Since both macrocysts and microcysts are resistant stages, it is a reasonable hypothesis to assume they arose by adaptation. The fact that macrocysts are probably sexual zygotes further supports such an adaptive argument.

8. Multiple acrasins also seem most easily interpreted as an evolutionary mechanism which has led to diversity and species formation. The difficult point to understand is how, biochemically, a species can change from one acrasin chemotaxis system to another.

Finally, if cellular slime molds are contrasted to larger, conventional organisms such as flowering plants or vertebrates, the slime molds have fewer constraints because of their small size and brief development. They are less insulated from their environment so that selection can play a constant and direct role of shaping almost all parts of their development. The one evolutionary feature they share with larger organisms is the important role of heterochrony in producing differences between species.

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## RAPID COMMUNICATION

### Macrocyst Formation in *Dictyostelium discoideum*: Mating or Selfing?

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**ABSTRACT** It is known from previous work that macrocysts are formed when the two strains of *Dictyostelium discoideum* (NC4 and V12) are mixed. By culturing NC4 and V12 amoebae together in various ratios and at several densities, and in experiments using vitally stained cells, we have shown that the number of macrocysts formed is directly related to the number of V12 (but not NC4) cells present and that macrocysts may occur without sexual fusion.

A starved population of *Dictyostelium discoideum* amoebae can either develop into asexual fruiting bodies or form macrocysts, depending upon a number of conditions including the appropriate environmental cues (Blaskovics and Raper, '59; Nickerson and Raper, '73), and whether or not amoebae of the opposite mating type are present (Clark et al., '73; Erdos et al., '73). The morphological sequence leading to macrocyst formation in *D. discoideum* has been described in some detail by Wallace ('77) and O'Day and Lewis ('81). Briefly, hundreds of amoebae aggregate to form tight cell clusters. Within an aggregate resides the cytophagic giant cell, the putative zygote. A primary wall is secreted around the aggregate transforming it into a precyst. The giant cell proceeds to engulf all of the amoebae in the precyst. In addition to the primary wall, secondary and tertiary walls, rich in cellulose, are formed around the macrocyst thus producing a well-protected, resistant cyst (Erdos et al., '72; Filosa and Dengler, '72; Nickerson and Raper, '73).

Because of the obvious importance of genetic studies, it is desirable to know if the two strains, NC4 and V12, of *D. discoideum*, which produce macrocysts when brought together, are producing zygotes or reproducing asexually possibly by selfing. The extremely low germination efficiency of *D. discoideum* macrocysts (<2%; Wallace and Raper, '79) does not allow one to show that all macrocysts are the results of zygote formation. In this study it is shown that there is macrocyst production without cell fusion of the two mating types. The question of whether there also may be some sexual zygote formation must wait for further analysis.

#### MATERIALS AND METHODS

##### *Growth and maintenance of stock cultures*

Stock cultures of *D. discoideum* strains NC4 and V12M2 were maintained on *Escherichia coli* B/r as a food source on nutrient agar plates (per liter distilled H<sub>2</sub>O: 10 g peptone, 10 g dextrose, 0.381 g Na<sub>2</sub>HPO<sub>4</sub>, 0.45 g KH<sub>2</sub>PO<sub>4</sub>, 20 g difco agar) at 22°C. Stocks were recultured weekly. For long-term storage, spores were lyophilized.

##### *Formation of giant cells and macrocysts*

Spores of NC4 and V12 were washed and heatshocked according to the method of Cotter and Raper ('68) and plated separately on nutrient agar with *E. coli* B/r. Synchronously developing amoebae could be obtained by harvesting such growth plates 12–18 h after inoculation and separating amoebae from *E. coli* B/r by differential centrifugation (Bonner, '47). Such NC4 and V12 amoebae, when cultured together on nonnutrient agar and incubated in the dark at 24 ± 1 °C, would produce giant cells in approximately 24 h and macrocysts in a few days.

##### *Scoring cultures*

The number of giant cells present in ten fields (totalling 17.4 mm<sup>2</sup>) were counted 24 h after incubation of NC4 and V12 together. Macrocysts were scored by counting the number per dissecting microscope field (7 mm<sup>2</sup>) of at least ten random fields in a Petri dish (86 mm<sup>2</sup> in diameter) and averaging these numbers. For both macrocyst and giant cell experiments, at least four replicates of each treatment were done and experiments were performed 2–4 times.

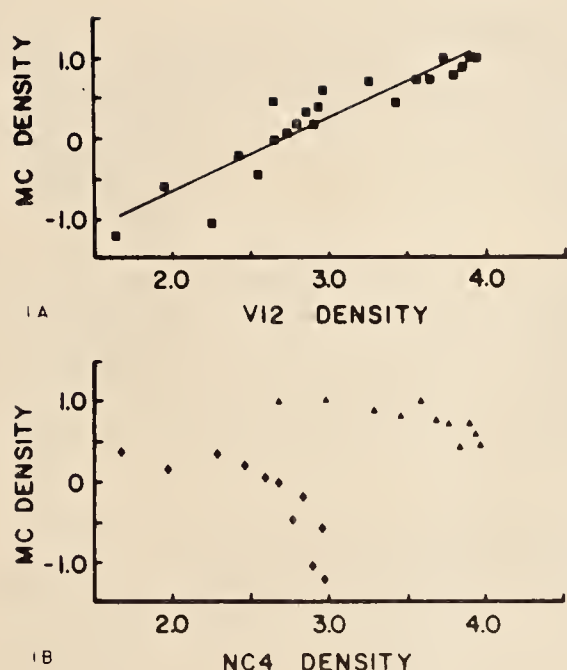


Fig. 1. Relationship between macrocyst density and cell density. A) Linear regression of the log of macrocyst density vs the log of V12 density ( $r = 0.92$ ;  $P < 0.01$ ). The first 11 points represent a total cell density of  $9 \times 10^2$  cells/mm<sup>2</sup> (NC4 + V12) and the next 11 points represent a total cell density of  $9 \times 10^3$  cells/mm<sup>2</sup>. B) Log-log plot of macrocyst density vs NC4 density. (◆) Total cell density equals  $9 \times 10^2$  cells/mm<sup>2</sup>; (▲) total cell density equals  $9 \times 10^3$  cells/mm<sup>2</sup>. Macrocyst densities ranged from 0.06 to 9.75/mm<sup>2</sup> and NC4 or V12 cell densities from 44 to 9,200 cells/mm<sup>2</sup>.

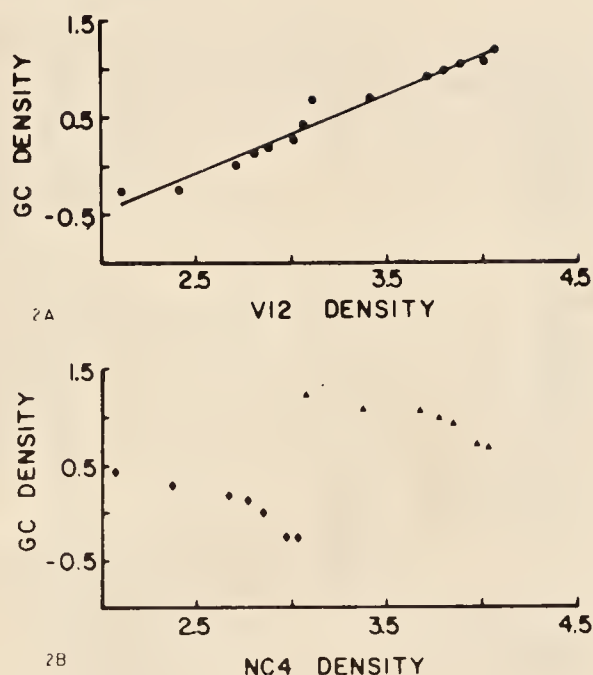


Fig. 2. Relationship between giant cell density and cell density. A) Linear regression of the log of giant cell density vs log of V12 density ( $r = 0.98$ ;  $P < 0.01$ ). The first seven points represent a total cell density of  $1 \times 10^3$  cells/mm<sup>2</sup> and the next seven points represent a total cell density of  $1 \times 10^4$  cells/mm<sup>2</sup>. B) Log-log plot of macrocyst density vs NC4 density. (◆) Total cell density equals  $1 \times 10^3$  cells/mm<sup>2</sup>; (▲) total cell density equals  $1 \times 10^4$  cells/mm<sup>2</sup>. Giant cell densities ranged from 0.60 to 16.4/mm<sup>2</sup> and NC4 or V12 cell densities from 120 to 11,000 cells/mm<sup>2</sup>.

## RESULTS

### Cell density and cell ratio experiments

If macrocyst formation depends upon the fusion of cells of opposite mating type, then at a constant cell density, a 50:50 mix of cells of opposite mating type should produce the maximum number of macrocysts. By mixing the two strains in different ratios at different densities, it is unequivocally clear that a 50:50 ratio between strains is not optimal; rather, the number of macrocysts varies with the number of V12 cells (Fig. 1A) and is independent of the number of NC4 cells (Fig. 1B).

It is possible that the reason macrocyst formation depends so critically upon V12 cells is that V12 amoebae are required at a stage of macrocyst development after fusion between cells to form giant cells. In this case, the formation of giant cells could still be optimal at a 50:50 ratio of the opposite mating types. Experiments were performed in which giant cells were counted before they became encased in macrocysts. As can be seen from Figure 2A

and B, the result is the same as that obtained in the macrocyst experiment. Note that low cell densities the number of giant cells almost equals the number of macrocysts (if Fig. 2A is superimposed over Fig. 1A), whereas at high cell densities, some of the giant cells never reach a macrocyst stage. This loss of giant cells at high cell densities may be due to phagocytosis of giant cells by other giant cells as has been demonstrated in *D. mucoroides* (Fukui, '76).

This phenomenon can be shown in another way. If all giant cells are indeed zygotes formed by the union of one NC4 and one V12 cell, one would expect that cell mixtures in which 10% of the cells were NC4 mixed with 90% V12 cells, and the reciprocal (90% NC4 and 10% V12), should yield equal numbers of giant cells since the probability of interstrain collision is the same in both of these mixtures. Instead, there are many more giant cells present in mixes of 90% V12 and 10% NC4 than in the reciprocal situation. Therefore, some of the giant cells produced under these conditions



TABLE 1. Giant cell formation at different cell densities and NC4:V12 ratios

NC4:V12 Ratio	Total cell density	Number of giant cells <sup>1</sup>
10:90	$1.2 \times 10^4$ cells/mm <sup>2</sup>	285.25 $\pm$ 32.76 <sup>2</sup>
20:80	$1.2 \times 10^4$ cells/mm <sup>2</sup>	212.00 $\pm$ 17.21
40:60	$1.2 \times 10^4$ cells/mm <sup>2</sup>	195.50 $\pm$ 35.03
50:50	$1.2 \times 10^4$ cells/mm <sup>2</sup>	166.00 $\pm$ 26.61
60:40	$1.2 \times 10^4$ cells/mm <sup>2</sup>	146.75 $\pm$ 16.76
80:20	$1.2 \times 10^4$ cells/mm <sup>2</sup>	90.25 $\pm$ 22.00
90:10	$1.2 \times 10^4$ cells/mm <sup>2</sup>	84.25 $\pm$ 10.98
10:90	$1.2 \times 10^3$ cells/mm <sup>2</sup>	47.50 $\pm$ 6.03
20:80	$1.2 \times 10^3$ cells/mm <sup>2</sup>	32.75 $\pm$ 4.13
40:60	$1.2 \times 10^3$ cells/mm <sup>2</sup>	26.25 $\pm$ 5.12
50:50	$1.2 \times 10^3$ cells/mm <sup>2</sup>	23.75 $\pm$ 3.71
60:40	$1.2 \times 10^3$ cells/mm <sup>2</sup>	17.75 $\pm$ 4.03
80:20	$1.2 \times 10^3$ cells/mm <sup>2</sup>	10.00 $\pm$ 2.92
90:10	$1.2 \times 10^3$ cells/mm <sup>2</sup>	10.00 $\pm$ 1.68

<sup>1</sup>Sum of ten fields (17.4 mm<sup>2</sup>).<sup>2</sup>Mean  $\pm$  standard error.

TABLE 2. Effect of extreme ratios of NC4 and V12 cell mixes on macrocyst production

NC4:V12 Ratio	No. MC/field <sup>1</sup>	MC/mm <sup>2</sup>	NC4/MC <sup>2</sup>
10 <sup>1</sup> :10 <sup>8</sup>	0 $\pm$ 0 <sup>3</sup>	0	—
10 <sup>1</sup> :10 <sup>7</sup>	0 $\pm$ 0	0	—
10 <sup>1</sup> :10 <sup>6</sup>	0.46 $\pm$ 0.35	0.068	0.025
10 <sup>1</sup> :10 <sup>5</sup>	0.95 $\pm$ 0.33	0.136	0.013
10 <sup>2</sup> :10 <sup>8</sup>	0.03 $\pm$ 0.03	0.004	4.3
10 <sup>2</sup> :10 <sup>7</sup>	0.35 $\pm$ 0.26	0.050	0.344
10 <sup>2</sup> :10 <sup>6</sup>	1.48 $\pm$ 0.81	0.211	0.082
10 <sup>2</sup> :10 <sup>5</sup>	2.50 $\pm$ 1.09	0.357	0.048
10 <sup>3</sup> :10 <sup>8</sup>	0 $\pm$ 0	0	—
10 <sup>3</sup> :10 <sup>7</sup>	0.93 $\pm$ 0.25	0.133	1.29
10 <sup>3</sup> :10 <sup>6</sup>	4.93 $\pm$ 1.05	0.704	0.244
10 <sup>3</sup> :10 <sup>5</sup>	3.00 $\pm$ 0.56	0.429	0.401
10 <sup>4</sup> :10 <sup>8</sup>	1.05 $\pm$ 0.25	0.150	11.47
10 <sup>4</sup> :10 <sup>7</sup>	3.08 $\pm$ 0.52	0.440	3.91
10 <sup>4</sup> :10 <sup>6</sup>	4.78 $\pm$ 1.02	0.683	2.52
10 <sup>4</sup> :10 <sup>5</sup>	9.08 $\pm$ 0.78	1.297	1.33

<sup>1</sup>Number of macrocysts per field (7 mm<sup>2</sup>).<sup>2</sup>Number of NC4 amoebae per macrocyst.<sup>3</sup>Mean  $\pm$  standard error.

could not be the product of NC4 and V12 cell fusion (Table 1).

It is evident that no macrocysts will form among V12 cells in the absence of NC4. One can now ask the question: How many NC4 cells are needed to form one macrocyst (on the average). This may be shown by mixing the two strains at low densities and extreme ratios (Table 2). From this experiment we see that macrocysts are produced when a mixture contains few NC4 cells and many V12 cells. In fact, in many of the cell mixes, there is, on the average, less than one NC4 cell per macrocyst. The column that gives the ratio of NC4 cells to macrocysts (Table 2) shows that this ratio may be as low as 0.0127, which means that on the average, only one NC4 cell is needed to produce 79 macrocysts! Therefore there are many macrocysts formed that contain only V12 amoebae.

#### Vital dye experiments

Sternfeld and David ('82) have provided convincing evidence that neutral red and Nile blue sulfate are effective dye markers for *D. discoideum* in that the cells retain the dye in their vacuoles and there is no loss or diffusion of the dyes from one cell to another. Here these dyes have been used to mark the two strains, and then once the macrocysts are formed they are gently crushed open so that the strain origins of the giant cell and the endocytes (ingested amoebae) can be identified.

When the cell mixture involved equal portions of NC4 and V12 cells, the color of the amoebae inside the macrocyst was predominantly that of V12, although NC4 was clearly present and sometimes abundant. When the ratio of the strains was one NC4 cell to one hundred V12 cells, the macrocysts appeared to be

exclusively made up of V12 cells. If NC4 cells of such mixtures were stained (so that their presence would be evident) and the nascent macrocysts were broken open liberating the endocytes, in many cases there was no evidence of NC4 cells.

#### DISCUSSION

It had been thought from the earlier work of Machac and Bonner ('75) that extracts or exudates of NC4 cells were capable of inducing macrocyst formation, but these experiments could not be repeated; they only worked when a very few live NC4 cells were present among the V12 cells. This observation is now confirmed: A small number of NC4 cells are sufficient to stimulate V12 amoebae to produce macrocysts, and NC4 need not be part of the macrocyst. Our results are consistent with those of Robson and Williams ('81), who have also demonstrated differences in the capacity of NC4 and V12 amoebae to participate in macrocyst production.

Somehow, live NC4 cells must induce V12 amoebae to proceed on the macrocyst development pathway. Perhaps they must come into contact with the V12 cells and in this way effect their cell surface. Alternatively, cell contact per se might not be required but cells must be in close proximity so that short-range interactions necessary for development can take place (Gross et al., '82). The nature of this stimulus is now under investigation in our laboratory.

In conclusion, macrocysts can develop without fusion of cells of the two mating types. We intend to determine whether sexual fusion might occur in addition to this nonsexual macrocyst formation. Also, there is the question of whether or not there is true selfing—that is, whether two V12 cells form a diploid and undergo meiosis or if the whole process is carried out by haploid cells.

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# Chemical identity of the acrasin of the cellular slime mold *Polysphondylium violaceum*

(glorin/peptide/chemoattractant/chemotaxis/amoeba)

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**ABSTRACT** The aggregation chemoattractant (or acrasin) of *Polysphondylium violaceum*, a species of cellular slime mold that does not respond chemotactically to cAMP, has been identified. It was extracted and purified from aggregating amoebae, then analyzed for amino acid composition and by IR and mass spectrometry. The active molecule is *N*-propionyl- $\gamma$ -L-glutamyl-L-ornithine- $\delta$ -lactam ethyl ester ( $M_r$ , 327), which we have named *glorin*. The compound has been synthesized and shows normal chemotactic activity with the amoebae of *P. violaceum*.

It has been known since the work of Shaffer (1, 2) that the chemoattractant or acrasin of *Polysphondylium violaceum* differs from that of the larger species of *Dictyostelium*. In 1968 (3, 4), it was shown that the acrasin of *Dictyostelium* is cAMP and that this substance, even though secreted by *Polysphondylium* amoebae, does not serve as a chemoattractant for that species (5-7).

The search for the chemical identity of *P. violaceum* began in this laboratory in 1973. A preliminary characterization was published in 1976 (8). At that time, there was evidence that it was a small molecule and it was suggested that it might be a peptide with its amino and carboxyl groups completely blocked. This early proposal has turned out to be correct.

## MATERIALS AND METHODS

**Glorin Collection.** The basic method of collection of the *P. violaceum* acrasin used was that devised by Francis (9) for *Polysphondylium pallidum*. Late vegetative amoebae were washed and placed on nonnutrient agar. When they were in full aggregation, 40% ethanol was poured over each Petri dish to inhibit the acrasinase and this ethanol solution was collected and concentrated.

Here are the details of the process. *P. violaceum* strain 1 was grown with *Escherichia coli* strain B/r in 40 large Petri dishes (15 × 150 mm) at 22°C for 40 hr in diffuse light on buffered agar/1% peptone/1% dextrose. The amoebae were harvested in 1% standard salt solution (10) and washed three times by centrifugation (5 min at 150 × *g*). The pellets of amoebae were diluted in 50% standard salt solution to produce a suspension of  $6 \times 10^6$  cells/ml. Fifteen milliliters of this suspension was placed in each of 150 large Petri dishes (15 × 150 mm) containing 2% nonnutrient agar and, after the amoebae had settled, the supernatant was carefully poured off and the plates were allowed to drain. All excess liquid was removed with filter paper and the plates were dried without covers for 10 min and then covered. After about 2 hr, when the amoebae were actively streaming into aggregations, each Petri dish was rinsed with 10 ml of 40% ethanol, which was decanted, and then rinsed again with

fresh 40% ethanol (10 ml over four successive Petri dishes). The final collection (2,000 ml) was centrifuged at  $16,300 \times g$  for 10 min, and the supernatant boiled down to about 50 ml and stored at -15°C.

**Chemotaxis Assay.** Chemotaxis was tested as described (8, 11) with two modifications. First, a 2- $\mu$ l drop of amoebae was placed directly on the agar instead of on a square of cellophane. If there was acrasin in the agar, the amoebae moved out more rapidly than in the control, presumably because the stable acrasinase removed the acrasin in the vicinity of the drop and the cells moved directly outward toward the higher concentration of acrasin beyond the drop. The second modification was that, instead of scoring the distance the cells moved out, the pattern of the response was scored qualitatively (Fig. 1). This visual scoring was found to be a more convenient index of high activity. *P. violaceum* amoebae are at the right stage of aggregation competence for a very restricted period of time and, immediately after they have passed their brief aggregation-sensitivity peak, the cells tend to reverse themselves and move back toward the new centers that have formed inside the original drop and begun to secrete their own glorin. Thus, the distance of outward movement from the drop is extremely variable, even in successive bioassays testing the same quantity of glorin. For this reason, we always ran two controls: (i) no glorin and (ii) a standard concentration that gave strong activity; only in this way could we be certain that the assay itself was working correctly.

**Purification of Glorin.** The purification procedure for 40 batches (150 Petri dishes each) of crude extract is summarized in Table 1. The extract was first filtered through a column of QAE-Sephadex (hydrochloride form) prepared with water to remove acidic impurities, which was washed with 2 column vol of water. The filtrates and washings of all the batches were combined and concentrated under reduced pressure to 30 ml. The concentrate was mixed with 200 ml of ethanol and the resulting precipitate was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure and the residue was dissolved in 50% ethanol for the column chromatography of step 2.

A Pharmacia K column with Tygon tubing and a Buchler peristaltic pump were used in steps 2-5. Step 6 was done on a Cheminert type LC column (Applied Science Division, State College, PA) with Viton tubing. In steps 7-9, an organic solvent-resistant column (Reliance Glass Works, Bensenville, IL; no. R-2790-500) with Teflon tubing was used, and solvent delivery was by gravity. In each step, fractions of the eluate were tested for chemotactic activity and the absorption spectrum (210-320 nm) was examined. Because the target compound does not absorb significantly above 210 nm, the decrease of absorption in

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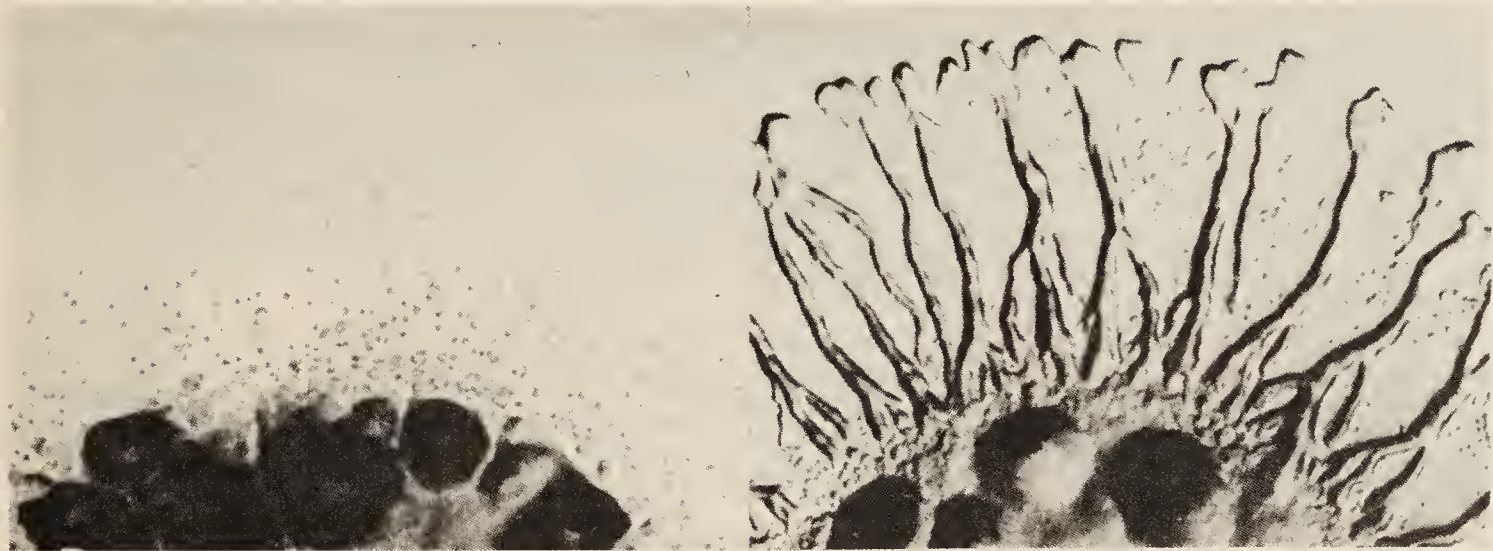


FIG. 1. Chemotaxis test for glorin. (Left) Control with no acrasin. (Right) A maximal response to acrasin in the agar; all the amoebae are incorporated into very strong streams. (This is recorded as a + + + + response; a + + + response is one in which there is a strong sunburst of streams moving out through a dense front of amoebae; + + indicates a dense front with some streams; and a + response is one in which there are no streams, yet the amoebae are moving out in a dense front.)

the region of this wavelength at each step was a convenient indicator of the progress of purification. Thus, the values of total absorbance ( $A_{1\text{cm}} \times \text{total volume in ml}$ ) at 250 nm for steps 2–9 were 230, 140, 7.8, 3.7, 0.75, 0.25, and 0.07, respectively.

Absolute ethanol (Publicker Chemical, Greenwich, CT) was used without further purification for harvesting; that used in chromatography was glass distilled before use. Acetone and acetonitrile were both spectrophotometric grade (Aldrich). Amino acid analyses were carried out by Sequemat (Watertown, MA) after hydrolysis with 5.7 M HCl at 110°C for 24 hr. The IR spectrum was measured by Sadtler Research Laboratory (Philadelphia), and mass spectrometry was done at the Mass Spectrometry Facility (Massachusetts Institute of Technology).

**Synthesis of Glorin.** The synthesis was carried out at Peninsula Laboratories (San Carlos, CA), by Ding Chang and K. Channabasavaiah. L-Glutamic acid  $\gamma$ -benzyl ester was first pro-

pionated with propionic anhydride and then esterified by treatment with thionyl chloride followed by ethanol, yielding propionyl-L-glutamic acid  $\alpha$ -ethyl- $\gamma$ -benzyl diester. Treatment of the diester with HF gave propionyl-L-glutamic acid  $\alpha$ -ethyl ester. This compound was condensed with L-ornithine- $\delta$ -lactam (12) by the mixed anhydride procedure with isobutyl chloroformate and *N*-methylmorpholine. The product was purified by column chromatography on Sephadex LH-20 using acetonitrile/water, 9:1 (vol/vol) as the eluent.

RESULTS

**Identification of Glorin.** Glorin has certain properties that limit the method of purification. (i) As reported (8), it is non-ionizable. (ii) On silica gel columns, glorin forms a very broad band with a variety of eluents and almost 50% of the activity is

Table 1. Purification of glorin by column chromatography

Step	Conditions	Total activity (A)*	Dry weight (B), mg	Specific activity, A/B
1	(Crude extracts from 40 batches) Filtration through QAE-Sephadex, 2.5 × 10 cm, 2 columns	20,000	4,000	5
2	Sephadex LH-20, 2.5 × 82 cm, 50% ethanol, 8 columns	15,000	150	100
3	Repeat of step 2, 3 columns	12,000		
4	Bio-Gel P-30 (100–200 mesh), 1.6 × 90 cm, 50% ethanol, 2 columns	10,000	6	1,670
5	Repeat of step 4, 2 columns	9,000		
6	Sephadex LH-20, 9 mm × 68 cm, 55% ethanol, 1 column	8,000		
7	Sephadex LH-20, 8 mm × 90 cm, 55% ethanol, 1 column	6,000	0.5	12,000
8	Sephadex LH-20, 8 mm × 83 cm, acetone/water, 8:2 (vol/vol), 1 column	4,500	0.220	20,500
9	Sephadex LH-20, 8 mm × 85.5 cm, acetonitrile/water, 8:1 (vol/vol), 1 column	2,500	0.092†	27,200

\*Total activity is given in activity units, where 1 unit corresponds to an activity of + + + in the chemotaxis assay (see Fig. 1).

† A colorless resin.



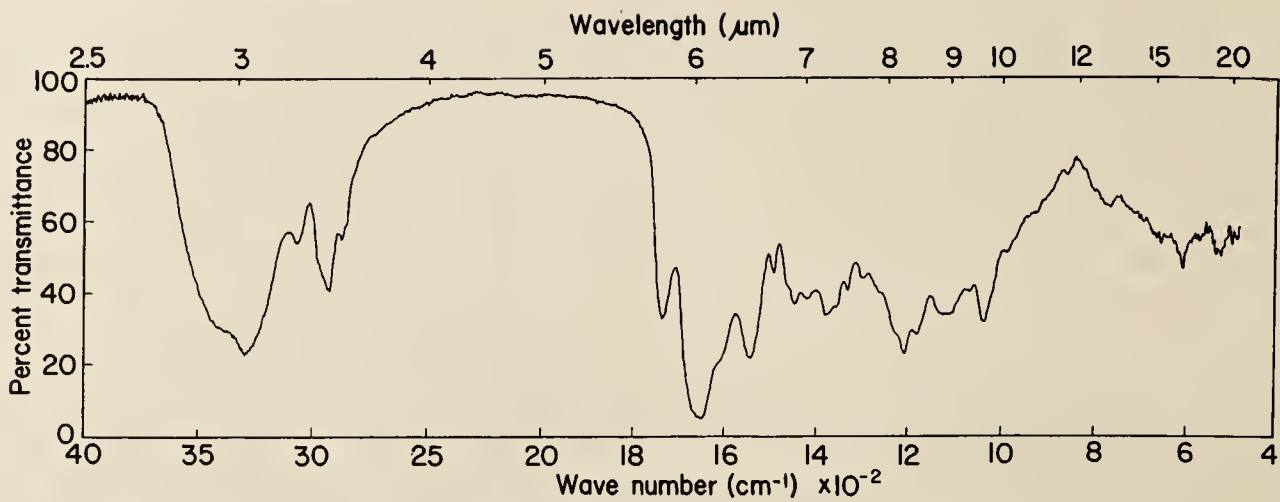


FIG. 2. Fourier transform IR spectrum (18.4 μg of glorin in a KBr micropellet).

lost. (iii) With Sephadex G-25 and other gels and an aqueous eluent, there is a very large loss of activity (>90%) despite the fact that glorin is stable when dissolved and left standing in water. The most successful method is the use of a Sephadex LH-20 column and an eluent that contains at least 50% organic solvent.

Purified glorin does not show an absorption peak above 210 nm in either the UV or visible region; there is some absorption at 210 nm, but it falls off steeply and becomes negligible above 260 nm. The IR spectrum resembles those of proteins and polypeptides (e.g., casein or gelatin), suggesting that glorin is probably a peptide (Fig. 2). The only significant difference is a peak at 1,739 cm<sup>-1</sup> that may be due to the C=O stretching vibration of an ester group in the glorin molecule.

Amino acid analysis of 9.2 μg of purified glorin showed, after acid hydrolysis, only two amino acid residues: 29.5 nmol of glutamic acid and 27.3 nmol of ornithine. If one assumes that the molecule contains 1 mol each of these two substances, the molecular weight is calculated to be 312 (from the amount of glutamic acid) or 337 (from the amount of ornithine).

Mass spectrometry using ionization techniques of field desorption and fast-atom bombardment showed that the molecular weight of this compound is 327. Furthermore, from the high-resolution fast-atom-bombardment mass spectrum, it was evident that the elemental composition of the M+1 species is C<sub>15</sub>H<sub>26</sub>N<sub>3</sub>O<sub>5</sub> (observed mass, 328.187265; calculated mass, 328.18724). Therefore, the elemental formula of glorin is C<sub>15</sub>H<sub>25</sub>N<sub>3</sub>O<sub>5</sub>.

The fragmentation pattern of the low-resolution electron-im-

pact-ionization mass spectrum (Fig. 3) suggests the presence of an ethyl ester group (M-46 peak) and, from the elemental formula and the presence of the two amino acid residues, it can be deduced that there must be a propionamide group (CH<sub>3</sub>CH<sub>2</sub>CONH). Both of these groups were confirmed by the high-resolution mass spectral data shown in Table 2 (M-C<sub>2</sub>H<sub>5</sub>OH, 281.13718; M-COOC<sub>2</sub>H<sub>5</sub>, 254.15013; M-C<sub>2</sub>H<sub>5</sub>CONH<sub>3</sub>, 253.11913).

Therefore, the molecule of glorin consists of one glutamic acid residue [COCH<sub>2</sub>CH<sub>2</sub>CH(NH)CO], one ornithine residue [NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH(NH)CO], one C<sub>2</sub>H<sub>5</sub>O group that is bound to one of the three C=O groups of the amino acid residues, and one C<sub>2</sub>H<sub>5</sub>CO group that is bound to one of the three NH groups in the amino acid residues. Based on the elemental formula, the two C=O groups and two NH groups of the amino acid residues remaining unbound must exist in the form of two

Table 2. High-resolution mass spectral data of glorin obtained by electron-impact ionization

Mass		Elemental composition
Observed	Calculated	
70.06655	70.06567	C <sub>4</sub> H <sub>8</sub> N
84.04767	84.04494	C <sub>4</sub> H <sub>8</sub> NO
85.05150	85.05276	C <sub>4</sub> H <sub>7</sub> NO
85.07801	85.07657	C <sub>4</sub> H <sub>8</sub> N <sub>2</sub>
98.06140	98.06059	C <sub>5</sub> H <sub>8</sub> NO
99.06669	99.06842	C <sub>5</sub> H <sub>9</sub> NO
113.04941	113.04768	C <sub>5</sub> H <sub>7</sub> NO <sub>2</sub>
113.07318	113.07149	C <sub>5</sub> H <sub>9</sub> N <sub>2</sub> O
114.07823	114.07932	C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O
115.08814	115.08714	C <sub>5</sub> H <sub>11</sub> N <sub>2</sub> O
126.08097	126.07932	C <sub>6</sub> H <sub>10</sub> N <sub>2</sub> O
140.07147	140.07115	C <sub>7</sub> H <sub>10</sub> NO <sub>2</sub>
141.06763	141.06640	C <sub>6</sub> H <sub>9</sub> N <sub>2</sub> O <sub>2</sub>
153.10260	153.10279	C <sub>8</sub> H <sub>13</sub> N <sub>2</sub> O
156.08821	156.08988	C <sub>7</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>
158.08079	158.08172	C <sub>7</sub> H <sub>12</sub> NO <sub>3</sub>
169.09690	169.09771	C <sub>8</sub> H <sub>13</sub> N <sub>2</sub> O <sub>2</sub>
181.09880	181.09771	C <sub>9</sub> H <sub>13</sub> N <sub>2</sub> O <sub>2</sub>
196.10961	196.10860	C <sub>9</sub> H <sub>14</sub> N <sub>3</sub> O <sub>2</sub>
214.10760	214.10794	C <sub>10</sub> H <sub>16</sub> NO <sub>4</sub>
231.13386	231.13449	C <sub>10</sub> H <sub>15</sub> N <sub>2</sub> O <sub>4</sub>
253.11913	253.11883	C <sub>12</sub> H <sub>17</sub> N <sub>2</sub> O <sub>4</sub>
254.15013	254.15047	C <sub>12</sub> H <sub>20</sub> N <sub>3</sub> O <sub>3</sub>
281.13718	281.13756	C <sub>13</sub> H <sub>19</sub> N <sub>3</sub> O <sub>4</sub>
327.17452	327.17943	C <sub>15</sub> H <sub>25</sub> N <sub>3</sub> O <sub>5</sub>

The most intense peaks of mass >70 are listed. Resolution: M/ΔM, 12,000.

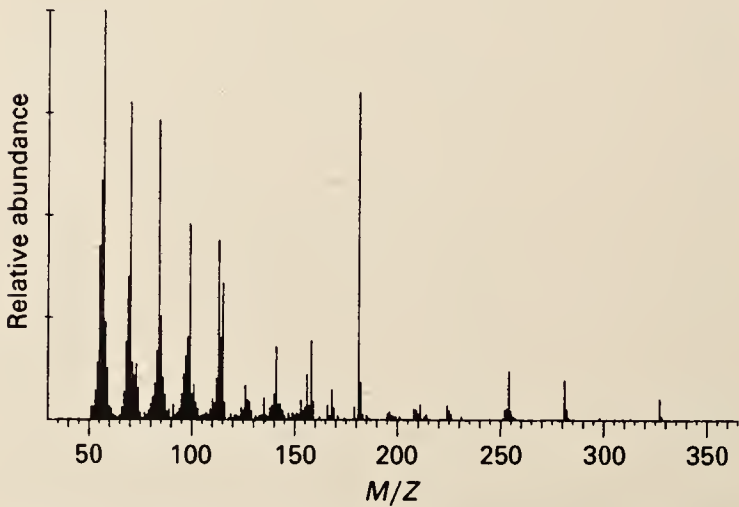


FIG. 3. Low-resolution mass spectrum of glorin by electron-impact ionization (70 eV).

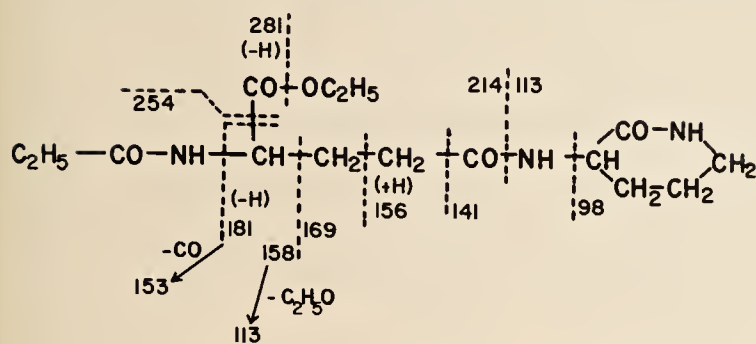


FIG. 4. Chemical structure of glorin. The major electron-impact-ionization mass spectral fragmentations are indicated.

peptide bonds—i.e., two NHCO groups.

It is possible to write 12 different structures knowing the above information. However, only one of those structures can satisfactorily explain the formation of the two fragments, 214.10760 ( $C_{10}H_{16}NO_4$ ) and 169.09690 ( $C_8H_{13}N_2O_2$ ). This structure (shown with some of its main fragmentations in Fig. 4) is wholly consistent with the high-resolution mass spectral data.

**Biological Activity of Glorin.** The substance shown in Fig. 4 was then synthesized from L-glutamic acid and L-ornithine, giving a colorless crystalline powder. Its mass spectrum was identical to the one obtained from purified natural glorin (Fig. 3).

Both the purified and the synthetic glorin were compared on the chemotaxis assay, this time using the quantitative test (11) in addition to the visual one that had been so useful in the purification (Fig. 1). Both the synthetic and the natural preparations of glorin showed identical activity with a peak at 10 nM, considerable activity at 100 nM, less at 1 nM, and none at 1  $\mu$ M. This is about 10 times the cAMP activity for *Dictyostelium discoideum*, where the activity peak using the same chemotactic test is 100 nM (13).

## DISCUSSION

Thus far, it is known that there are at least eight different acrasins in the cellular slime molds (14) but only one of these, cAMP, has been identified chemically. Folic acid and related substances serve as powerful attractants (15, 16) but they seem to act most effectively on vegetative amoebae rather than as an aggregation acrasin (13, 15). Recently, Konijn and co-workers (17) have shown that the acrasin of *Dictyostelium lacteum* is a pterin but not folic acid. Therefore, the structure of glorin reported here represents a different type of acrasin that has been fully identified.

There are a number of reasons why this discovery holds considerable promise. In the first place, the substance is a peptide. Like so many of the peptide attractants of leukocytes, it is also terminally blocked (18). The *N*-formylmethionyl peptides used for leukocyte chemotaxis do not attract *P. violaceum* amoebae (unpublished results), but the basic molecular similarity is striking despite the difference in specificity. Second, it must be remembered that, in the basic patterns of their life histories, the large *Dictyostelium* species that use cAMP for their acrasin are similar to *Polysphondylium*, which uses glorin. (*P. pallidum* is responsive to glorin and is presumed to have it as its acrasin, but this must be carefully checked.) This means that two entirely different signalling systems, including different acrasinases, receptor proteins, and so forth, do essentially the same thing and, therefore, the opportunity to do comparative signal-receptor physiology may be as rewarding as it has been with

different neurotransmitters. The third point of interest is that *Polysphondylium* also secretes cAMP (5, 6) which is known to be involved in differentiation, as is well established to be the case in *Dictyostelium* (refs. 19 and 20; for review, see ref. 21). Does this mean that in *Dictyostelium* cAMP performs two separate functions (and perhaps more) while in *Polysphondylium* the chemotactic system is chemically quite separate from the differentiation system? And finally, what is the relationship between glorin, cAMP, cGMP, the D factor of Hanna and Cox (22), and other key substances involved in development, including  $\text{NH}_3$  (23) and possibly differentiation-inducing factor, the stalk-forming morphogen of Town and Stanford (24). It is hoped that the study of glorin and its activities will open up new avenues of research in our attempt to understand development in molecular terms.

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# Chemical Signals of Social Amoebae

*Two cellular slime molds can coexist in the same soil and yet maintain their identity. They do so by emitting and responding to different chemicals, both of which have now been identified*

by John Tyler Bonner

In any bit of reasonably fertile soil two very small unicellular organisms are abundant: bacteria and amoebae. There may be millions of bacteria in a cubic centimeter of soil, and thousands of amoebae. The bacteria tend to be clumped near dead roots or dead insects, which supply them with nutrient, and the amoebae eat the bacteria. Each amoeba is about the size of a white blood cell, some 10 times longer than a bacterial cell. It crawls through the soil, engulfing bacteria it encounters, and every few hours it divides to form two daughter cells.

Prominent among the commonest kinds of soil amoebae are the social amoebae, otherwise known as the cellular slime molds. They feed as separate cells, like other amoebae, but once they have finished off all the bacteria in an area a trigger is set off within each cell that initiates their social phase. The cells stop their solitary wandering and begin to stream toward a central collecting point. Anywhere from a few hundred to 100,000 or so unicellular organisms come together to create a single, sluglike mass that has become a multicellular organism.

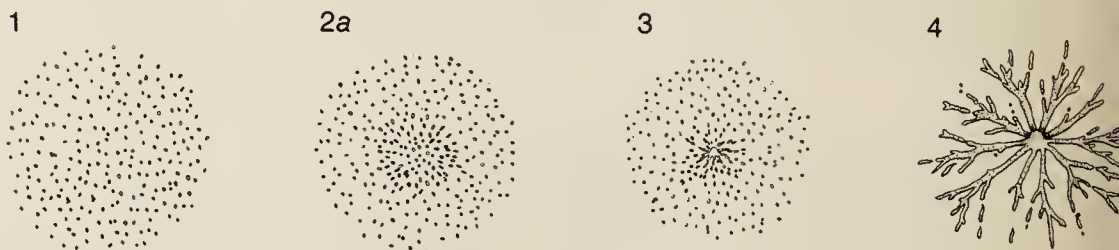
Then the cells begin to differentiate. A nipplelike tip appears at the front end of the mass, and inside the tip the beginning of a stalk takes shape. The stalk is a cellulose cylinder composed of swollen, rigid cells that ultimately die. The stalk builds upward as cells stream up the outside of it and pile onto the tip. When all movement has ceased, the mass of cells has become a fruiting body: a simple or branched stalk made up of dead cells encased in a cellulose sheath and capped by a little ball of spores. The spores are individually encapsulated amoebae, which become dispersed by water or by contact with a passing worm or insect. If the spore lands in a suitably warm and moist environment, it splits open, releasing a single amoeba that resumes the foraging phase of the slime-mold life cycle.

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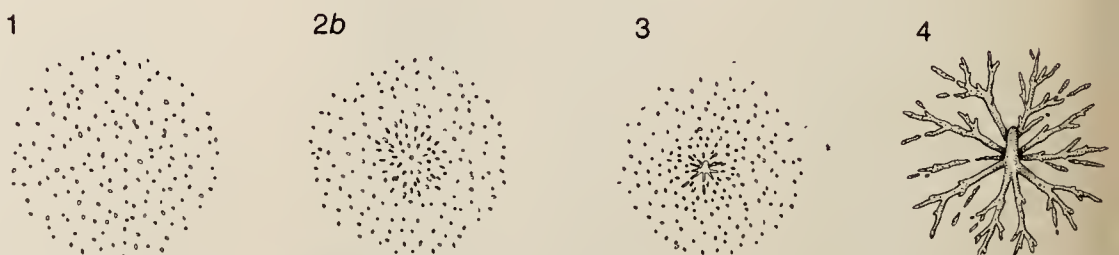
a particular aspect of the social amoebae's behavior. Often there are two or more species of social amoebae in the same bit of soil. When they aggregate, they aggregate separately: the cells of different species become oriented toward separate collection centers. The streams of cells heading for one center pass through, and ignore, streams head-

ed for another center, so that each species comes together at its own collection center and differentiates to form its characteristic fruiting body. How does it happen? Pieces of the answer have been emerging for some 40 years, and now it is finally possible to describe the segregation of the two species at the molecular level.

*DICTYOSTELIUM MUCOROIDES*



*POLYSPHONDYLIUM VIOLACEUM*



**LIFE CYCLE AND MORPHOLOGY** of two cellular slime molds, *Dictyostelium mucoroides* and *Polysphondylium violaceum*, are similar. They feed as individual amoebae (1) until lack of food (bacteria) triggers either a group of cells (2a) or a single cell (2b) to secrete a chemical substance, called an acrasin, that attracts surrounding amoebae (3). The cells stream toward a

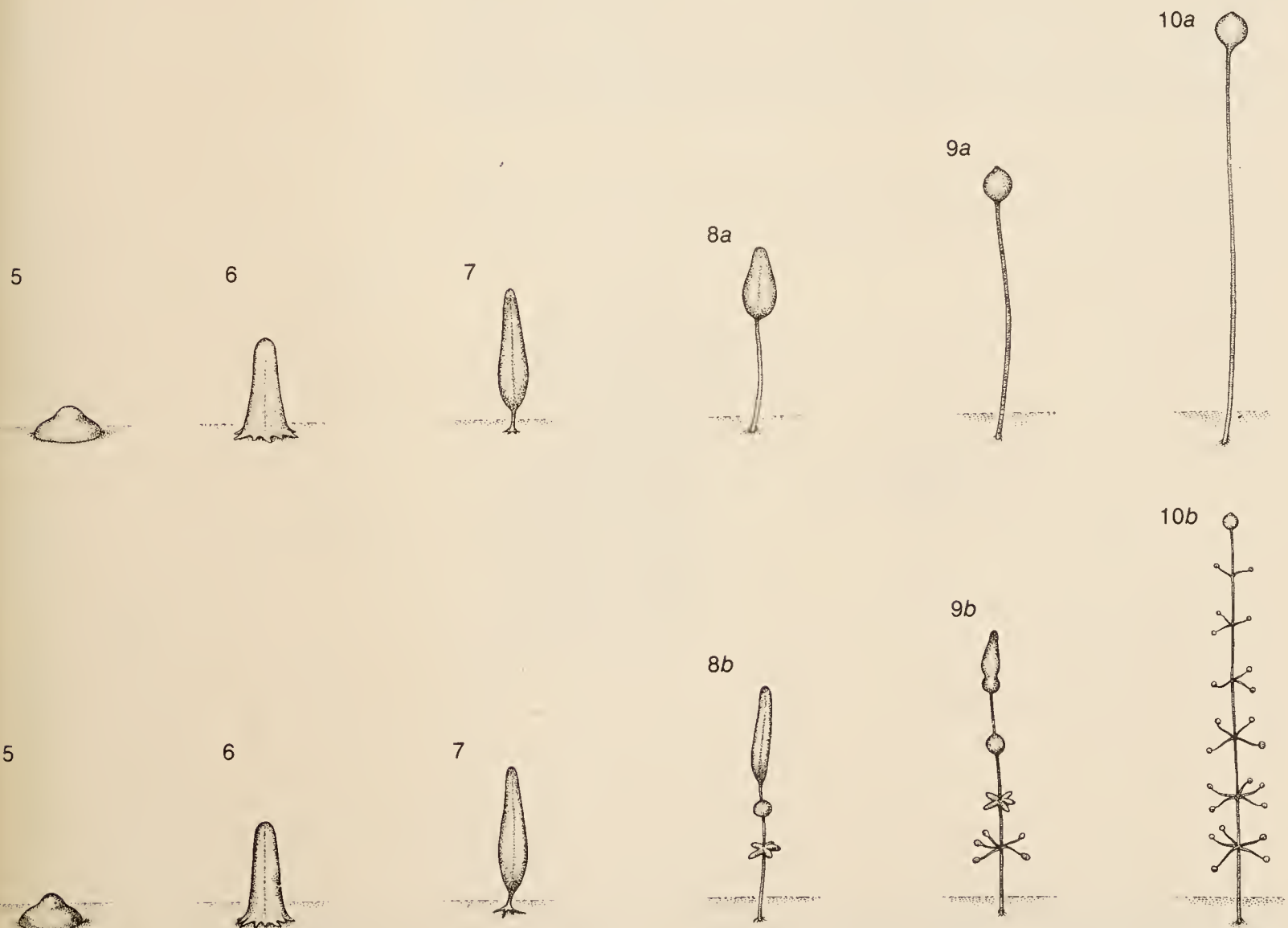
The segregation phenomenon was noted in 1940 by Kenneth B. Raper and Charles Thom, who were then working in the U.S. Department of Agriculture. They grew cultures of the bacterium *Escherichia coli* and inoculated a culture dish with two species of social amoebae, *Dictyostelium discoideum* and *Polysphondylium violaceum*. The amoebae multiplied and grazed their way outward from the point where each species had been inoculated; eventually the expanding populations were thoroughly mixed. Having consumed the available bacteria, the amoebae in the region of mixing began to aggregate. As Raper and Thom wrote, "they regularly aggregated to different centers with the converging streams of the two forms commonly overlapping.... It was obvious that the stimuli in different species differed qualitatively." Raper and Thom recognized why that had to be. If the two species are together in the same soil, they must have a way to avoid coming together in the same aggregate in order to remain distinct species. Raper and Thom could not know how the two stim-

uli differed because the nature of the stimuli was not then known.

At the turn of the century scientists studying cellular slime molds had assumed that the amoebae must be brought together by some form of chemotaxis, but in the 1940's accounting for a developmental process by chemical attraction was viewed with suspicion; some form of "contact guidance" was a much more popular explanation. In 1942 Ernest H. Runyon of Agnes Scott College made it clear, however, that contact among cells could not bring about aggregation. In a series of experiments over the next few years I was able to show that the central mass had to be emitting some diffusible attracting agent. It could have been heat, but a number of indications made it clear that the agent must be a chemical substance. I called the substance an acrasin. (Acrasia is the name of a witch in Edmund Spenser's *Faerie Queene* who attracted men and transformed them into beasts, and *Dictyostelium* is a member of the Order Acrasiales.)

Over the years studies in numerous laboratories, including our own at Princeton University, have revealed many details of the acrasin system. Starvation is the stimulus for the switch from the feeding state to the social one. It sets in motion a series of chemical events within the individual cells, which soon begin to secrete an acrasin. At the same time the amoebae synthesize and display on their surface a large number of specific protein molecules that serve as receptors for the acrasin. The cells also secrete an acrasinase (an enzyme that inactivates the acrasin) and an inhibitor of the acrasinase. The acrasin, the acrasinase and the inhibitor combine to form a complex control system that modulates aggregation as it proceeds. To take just one example, the acrasinase keeps the external acrasin level generated by an individual amoeba low enough so that the cell can sense acrasin emitted by other cells.

Aggregation begins differently in different species, as Brian M. Shaffer, who was then working at the University of Cambridge, found some years ago. In



central collection point and pile up to form a multicellular organism (4). A tip forms on the cell mass (5) and the cells begin to differentiate. Some of them form a rigid internal stalk (6, 7). The remainder are borne aloft on the stalk to become spores. In *Dictyostelium* there

is a single spherical mass of spores at the tip of a mature fruiting body (8a, 9a, 10a). In *Polysphondylium* there are whorls of small fruiting bodies in addition to an apical spore mass on the central stalk (8b, 9b, 10b). The spores become dispersed and split open, freeing amoebae.



*Polysphondylium* a single cell in the population suddenly rounds up and begins to secrete acrasin. Neighboring cells streak in to join it, oriented by the concentration gradient of the secreted acrasin. In *Dictyostelium*, on the other hand, a small group of cells (Shaffer called it a cloud) seems to initiate aggregation. These cells first become relatively immobile and then clump together, and the clump attracts the surrounding cells. In both species the aggregating amoebae often come inward in pulses. There seems to be a good reason: to establish an effective overall gradient of acrasin that will orient distant cells, a good-sized central mass is required. The pulses can orient cells before such a gradient is established. If one amoeba gives off a puff of acrasin and the puff diffuses out to neighboring cells, it hits each cell on one side first, filling more of the cell's receptors on that side than on other sides, and thereby orients the cell. The responding amoeba in turn emits a puff that attracts cells beyond it.

When chemotaxis was firmly established as the mechanism of aggregation, I argued at first that it was not important to know the chemical nature

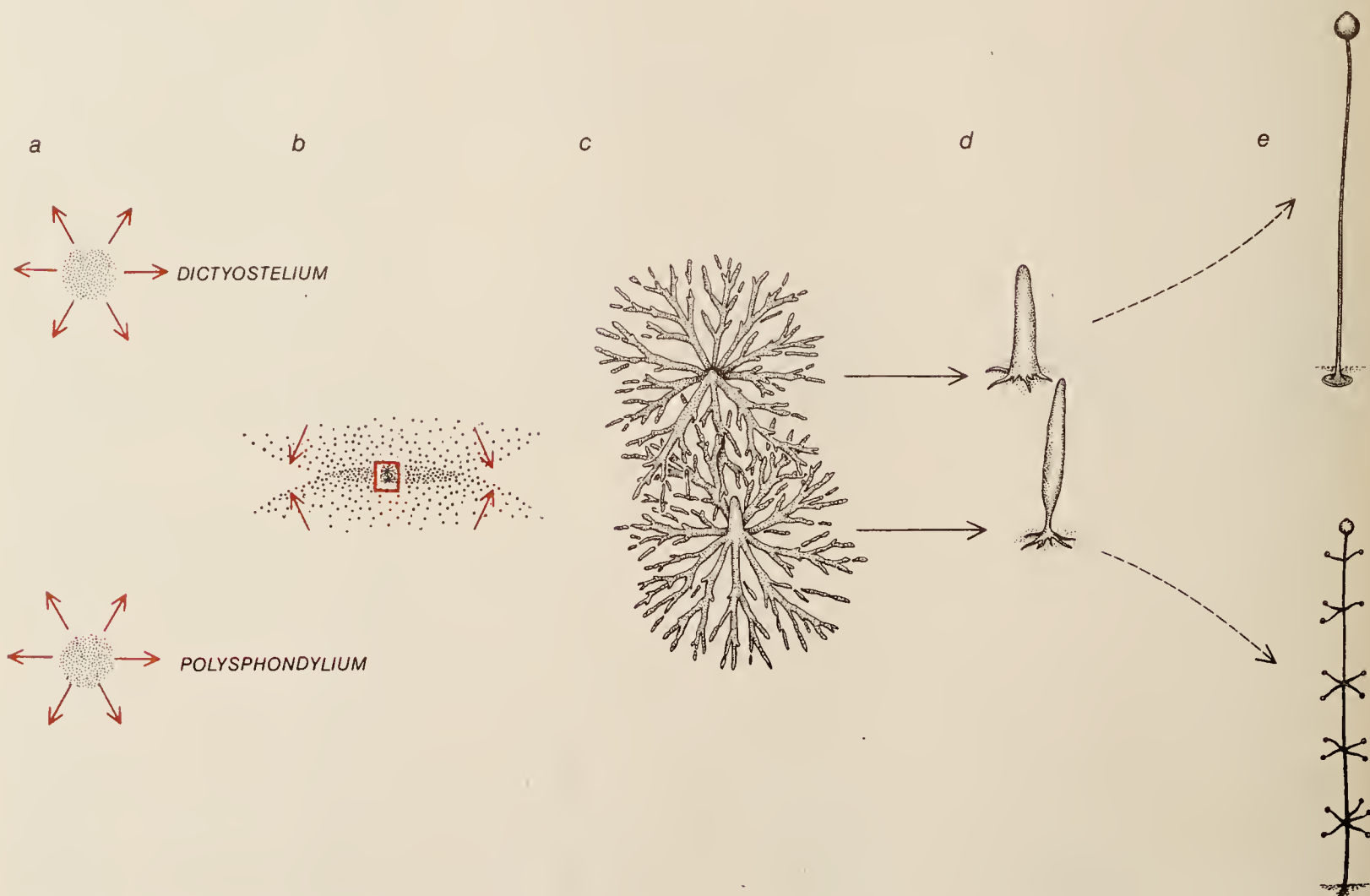
of the acrasins. I say this with some embarrassment because the fact is that the moment the chemical structure of a key substance in physiology or development is discovered, one goes from the dark ages to modern times. Certainly that was true in the case of the *Dictyostelium* acrasin. In any event we soon joined other laboratories in an effort to characterize its attractant. It took from 1947 to 1967. I have told the story in detail before [see "Hormones in Social Amoebae and Mammals," by John Tyler Bonner; SCIENTIFIC AMERICAN, June, 1969], and I shall only summarize it here.

The major difficulty was developing a convenient bioassay: a way to test the biological effect of successively purified substances. Such assays were finally developed by Theo M. Konijn (who is now at the University of Leiden) and by us. We both did preliminary work toward identifying a factor found in bacteria that seemed to attract amoebae. Konijn came to Princeton, and soon after he arrived David S. Barkley, who was then a graduate student, had an inspiration: Why not try cyclic adenosine monophosphate (cyclic AMP), a nucle-

otide that Earl W. Sutherland, Jr., of the Vanderbilt University School of Medicine had discovered and had shown to act in mammalian cells as a "second messenger," mediating between hormones and cell functions.

It worked, with remarkable potency, in our chemotaxis assays. We went on to show that *Dictyostelium* cells not only respond to a minute amount of cyclic AMP but also produce it. (Now, to be sure, it is well known that almost all cells make cyclic AMP; not producing it is the rarity.) More to the point, we found that during aggregation the amoebae secrete the nucleotide in particularly large quantities (a hundredfold increase) and also are about 100 times more sensitive to the cyclic AMP than they are at other times.

The discovery that cyclic AMP is the natural acrasin of *Dictyostelium* opened up new lines of investigation. Workers in laboratories all over the world have studied just how the nucleotide orients cells and have analyzed the activity of adenyl cyclase, the enzyme that catalyzes the synthesis of cyclic AMP, and of phosphodiesterase, the acrasinase for this acrasin; they have examined the



**TWO SLIME-MOLD SPECIES** aggregate separately, as Kenneth B. Raper and Charles Thom demonstrated in 1940 with this experiment. A culture of the bacterium *Escherichia coli* is inoculated with individual cells of *Dictyostelium discoideum* (top) and *P. violaceum* (bottom). The amoebae graze outward from the point of inoculation (a), be-

come mixed and begin to aggregate (b). When the aggregations are examined under the microscope (c), they are seen to be separate. Streams of amoebae of the two species head for two different collection centers and form separate organisms (d), which develop to form distinctive fruiting bodies that are characteristic of the two species (e).



cell-surface receptors for cyclic AMP and the interactions of all the elements of the system. In 15 years there has been great progress toward understanding how some aspects of the development of *Dictyostelium* are controlled, and the new knowledge has implications not only for the development of the cellular slime molds but also for development in general.

As soon as we knew that cyclic AMP attracts *Dictyostelium* cells we tried it on *Polysphondylium*. It had no effect, just as Raper and Thom might have predicted (and as Shaffer later had predicted). The two species have different acrasins. The ensuing effort to identify the *Polysphondylium* acrasin was very different from our *Dictyostelium* experience. There was no clever guess by a bright graduate student. We could not even find a good source of unpurified attractant; bacterial products and even human urine had been good sources of cyclic AMP. We tried a variety of things that are generally rich in hormones and other active substances—milk, peptones (mixtures of peptides), yeast extracts and many others—with no effect. We had no recourse but to set about extracting the material from aggregating amoebae. The moral is that one cannot be blessed with fantastic luck every time.

The first step was to find out how best to extract the acrasin from an aggregation of *Polysphondylium*. As we had learned working on *Dictyostelium*, the acrasinase secreted along with acrasin tends to break down the attractant as soon as it is secreted. We finally settled on a method devised by David W. Francis, who was then a postdoctoral fellow at Princeton and is now at the University of Delaware. It consists of dumping 40 percent ethyl alcohol directly on petri dishes containing cells in midaggregation, washing the dishes with more alcohol and then collecting the wash in a beaker. We boil off the alcohol and centrifuge off the precipitate that forms. A crude extract of acrasin is left in solution; it remains intact because the alcohol has denatured the acrasinase.

The remarkable thing is that the amoebae do not seem to be bothered by this 80-proof dunking; they go right on with their development after the alcohol has been removed. In order to collect as much crude extract of *Polysphondylium* acrasin as possible, over the years we have developed a routine that begins with 150 large petri dishes of aggregating amoebae, which cover every available surface in the laboratory. It has the disadvantage of stopping all other experiments for lack of space, but the laboratory does have a rather pleasant aroma. At first we used to think we could smell the acrasin, but it turned out to be the alcohol.



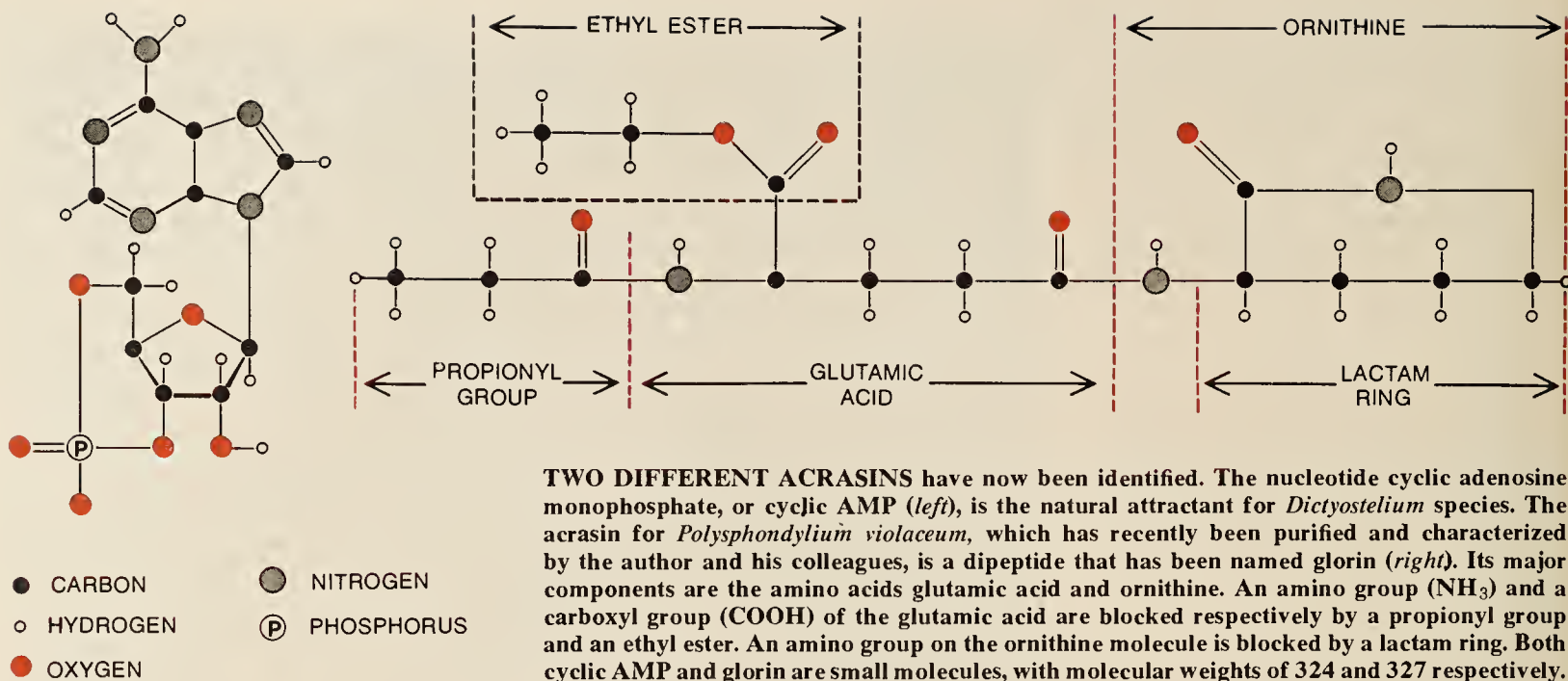
**SEPARATE AGGREGATION** of two species was recorded in this photomicrograph, made by Raper and Thom, in which the streams of aggregating amoebae are enlarged some 45 diameters. There is a small aggregation of *D. discoideum* (top) and a larger aggregation, with two dark centers, of *P. violaceum*. The inflowing streams of amoebae overlap, with the cells of each species apparently ignoring those of the other species as they head for their own center.

In the early days of this project, almost a decade ago, we sought the major characteristics of the *Polysphondylium* acrasin. This was done by Bernd Wurster (who is now at the University of Konstanz in Germany) and Pauline Pan (now with the Pennwalt Corporation). After more than two years of work we could say that the acrasin was a small molecule, with a molecular weight of less than 1,500 (estimated from its movement on a gel filtration column); that it was stable when heated; that it did not appear to be ionic in nature (that is, that the molecule was uncharged), and that it did not have any free amino or carboxyl groups, which are usually found respectively at the beginning and the end of a protein chain. Yet two enzymes—one protease and one peptidase—of a number we tried completely inactivated the acrasin, suggesting it was some kind of peptide (a short piece of a protein chain). Unfortunately this was not a conclusive finding because the enzyme preparations were impure; something quite secondary could have been playing a role. These were, however, neutral enzymes, which attack un-

charged polypeptides, and this was consistent with the observation that the acrasin molecule seemed not to be charged. We concluded that the *Polysphondylium* acrasin might be a peptide in which there was no free amino group, with some evidence that it incorporated what is called an ester bond.

Further analysis was difficult because in spite of our best efforts our purest extracts were very impure. It was at this point that our colleague Osamu Shimomura, who is now at the Marine Biological Laboratory in Woods Hole, Mass., became interested and took charge of all the chemistry, including the purifications. To give him enough material to work with we had to increase the number of harvests. Under the direction of Hannah B. Suthers we started an "acrasin factory": two days of the week were devoted entirely to harvesting the 150 large petri dishes of acrasin. This went on over three years, for a total of 96 harvests. In the first two years we were still experimenting, perfecting the method of purification. In the third year we made what turned out to be the final push to accumulate crude acrasin, and





40 collections gave us an initial four grams of very impure material. Shimomura put this through a series of nine purification steps involving three different kinds of gel filtration columns and numerous solvent systems. At the end we had 92 micrograms (millionths of a gram) of acrasin, about 98 percent pure.

The 92 micrograms of acrasin was divided into lots of about 10 micrograms that were sent away for different kinds of analysis. From the infrared

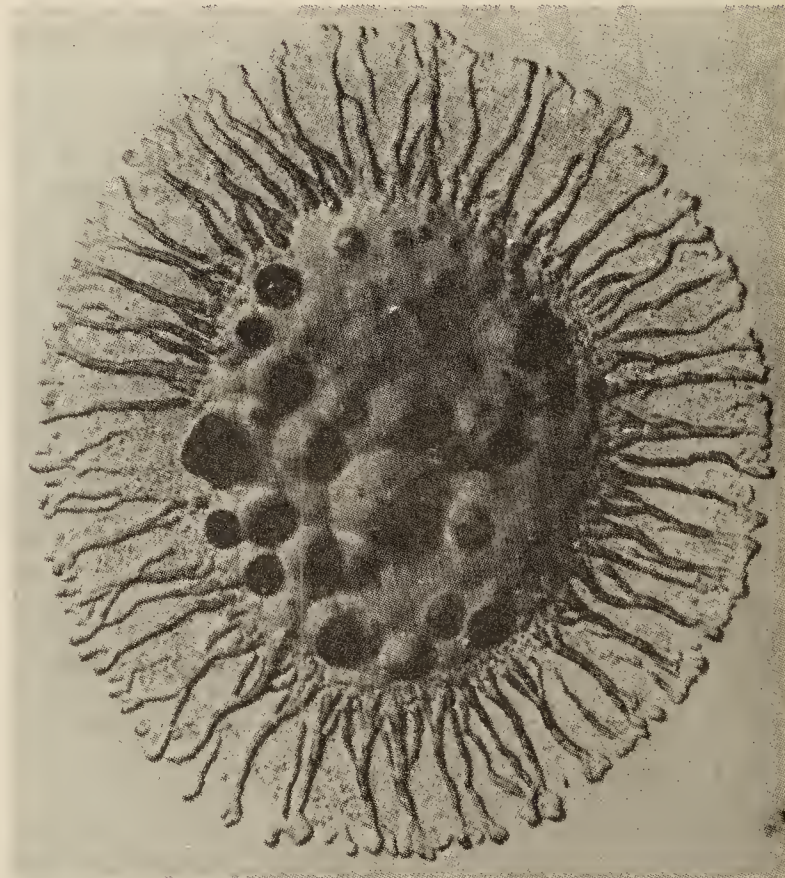
spectrum we learned that there was indeed the strong possibility of an ester group. Amino acid analysis confirmed an earlier finding that the molecule contained two amino acids, glutamic acid and ornithine (which is not one of the usual components of proteins), in roughly equal amounts. The excitement came from the results obtained by Catherine E. Costello of the mass-spectrometry facility at the Massachusetts Institute of Technology. Using the latest magic (ionization techniques of field de-

sorption and fast-atom bombardment, at both low and high resolution), it was possible in one final push to know the complete structural formula and the molecular weight. This is quite remarkable, considering that it comes from looking at a sample of about 10 micrograms of material.

The *Polysphondylium* acrasin turned out to be a dipeptide. There is a glutamic acid molecule with two side groups: a propionyl group, which blocks the amino end of the amino acid, and an eth-



**BIOASSAY** demonstrates that glorin is indeed the natural attractant for *P. violaceum*. When a large clump of amoebae is placed on a block of agar (left), all but a few of the cells stay in the clump. When a clump of amoebae is placed on a block of agar that has been impregnated



with synthetic glorin (right), the amoebae stream outward vigorously, attracted by the glorin. (The glorin directly under the clump is rapidly destroyed by an acrasinase, an inactivating enzyme secreted by the cells. *P. violaceum*'s acrasinase has not yet been identified.)

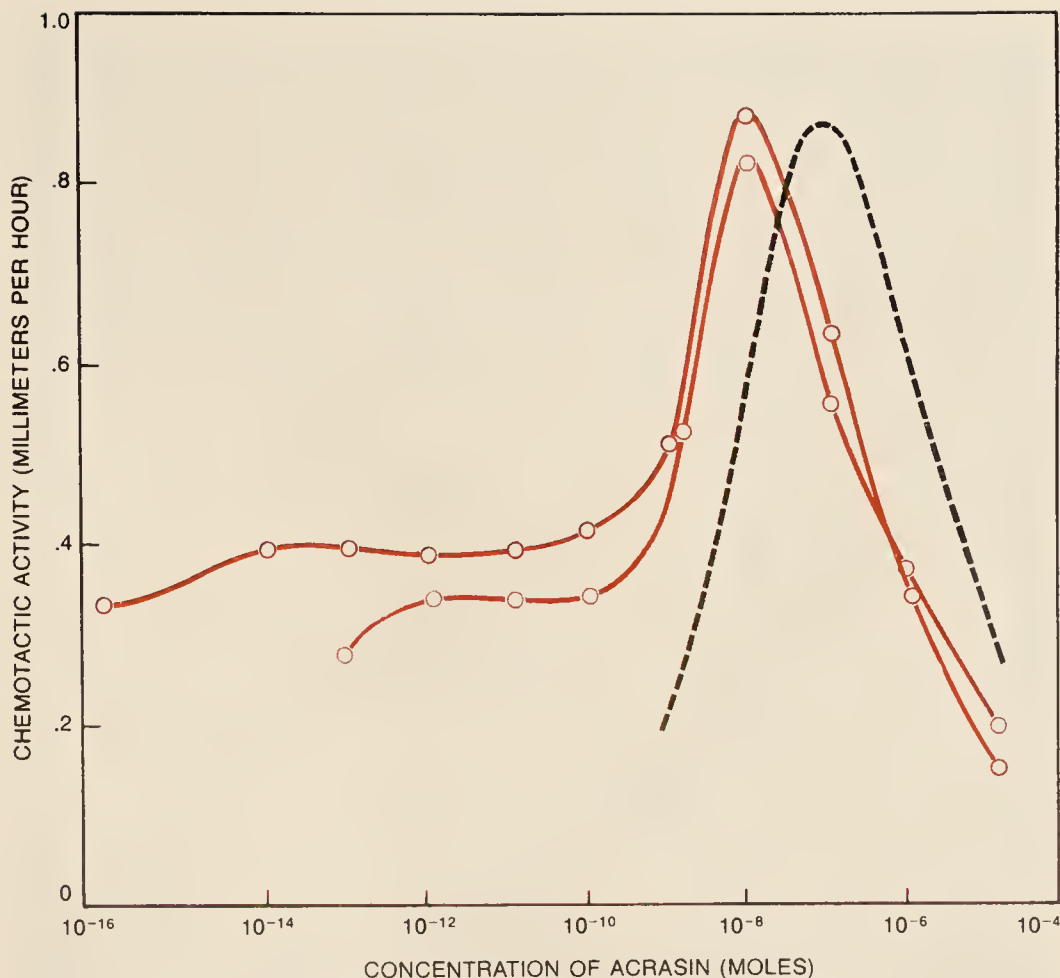


yl ester, which blocks the free carboxyl group. The glutamic acid is attached by a peptide bond to an ornithine, but the ornithine is closed into a lactam ring, which again blocks a free amino group. The molecular weight of the acrasin is 327. Because it consists of glutamic acid and ornithine, whose abbreviations are *Glu* and *Orn*, we decided to call the acrasin glorin.

To confirm that glorin is the naturally occurring acrasin of *Polysphondylium* we needed to have the dipeptide synthesized and then test the manmade version in our chemotaxis assay with *Polysphondylium* amoebae. We sent our glorin formula to Peninsular Laboratories, Inc., and after a month or so they delivered to us 50 milligrams of synthetic glorin. (We calculated that it would have taken more than 500 years to accumulate that much material by extraction and purification in our laboratory!) At M.I.T., Costello immediately showed that its mass-spectral properties were identical with those of the natural, purified glorin. At Princeton, with considerable anxiety, we undertook the bioassay.

The result was unequivocal. The synthetic glorin had enormous biological activity: when it was placed in agar surrounding a mass of test amoebae, they streamed in a most dramatic way [see bottom illustration on opposite page]. In a quantitative test we compared the synthetic glorin's ability to attract amoebae with that of the natural, purified glorin (the remains of our precious hoard) and found the two were essentially identical in their activity. When we compared the glorin curves with data showing the effect of cyclic AMP on aggregating *Dictyostelium* amoebae, it was clear that glorin works effectively at even more dilute concentrations than cyclic AMP does.

So much for the excitement of the chase. What can we now do with glorin? One thing is to work out all the details of the glorin aggregation system, including the identification of its receptors, the acrasinase and the inhibitor of the acrasinase. We also want to ask this question: What are the respective contributions of glorin and of cyclic AMP to differentiation? The reason for this interest is that it has been known since our early experiments with Konijn that cyclic AMP is present in *Polysphondylium*. Later, in our laboratory and in others, mounting evidence was found that cyclic AMP is involved in the differentiation of both stalk cells and spores in *Dictyostelium*, and there is similar evidence in *Polysphondylium*. There is even interesting work in *Dictyostelium* showing that cyclic AMP is directly involved in controlling the expression of some developmental genes. Recent work in laboratories in the Netherlands and the



**CHEMOTACTIC ACTIVITY** of glorin synthesized in the laboratory (dark color) is compared with the activity of the natural attractant (light color) purified from aggregating cells. The two curves are essentially identical, confirming the chemical analysis of the natural glorin. A similar curve (gray) shows the effect of cyclic AMP on aggregating cells of *D. discoideum*.

U.S. has provided convincing evidence that chemotaxis continues to operate in the cell mass during later stages of development: a cyclic AMP gradient within the multicellular organism orients the cells, thereby guiding the direction of the advancing tip. During these later stages of *Dictyostelium* development, then, cyclic AMP is involved in two things, chemotaxis and differentiation, at the same time and in the same place.

We want to find out if in *Polysphondylium* there is perhaps a separation of functions, with chemotaxis controlled by glorin and differentiation controlled by cyclic AMP. The relation of the two signaling systems may not be simple and separate; it is quite possible that they are mutually dependent on one another or that glorin is more directly involved in cell differentiation than we currently assume. Here lies a unique opportunity to compare two parallel developmental systems that have different chemical signaling systems. Among other things, it should provide a way to separate, isolate and identify the controls of the different developmental events.

Perhaps the main point is that *Dictyostelium* and *Polysphondylium* live side by side in the soil, are similar in appearance and go through remarkably similar stages of development. Yet they have

totally different signaling systems, one system based on a nucleotide and the other on a peptide. The only comparable situation is found in the body of animals, including human beings, where cyclic AMP acts in many signaling reactions, both hormonal and neural, and a number of peptides function in the nervous system as neurotransmitters. The presence of rival signal systems based on small nucleotides and on peptides is a very general biological phenomenon, not just a slime-mold curiosity.

Some broad evolutionary questions arise from the fact that these two signaling systems are so different. I pointed out above that Raper and Thom's experiments seemed to imply that by responding to two different stimuli two species living in the same place can keep from intermingling and thereby losing their identity. The problem extends beyond two species. James C. Cavender of Ohio University and Raper, who have spent much time collecting slime molds all over the world, have shown that in some temperate and tropical environments as many as from five to eight species may coexist. It is not necessary for all of them to have different acrasins to keep separate. As Raper and Thom showed in their original paper, there can also be "cell-surface incompatibility": some





**STALKS AND SPORE MASSES** of *Polysphondylium pallidum* (which, like *P. violaceum*, secretes and responds to the acrasin glorin) are enlarged 16 diameters in a photomicrograph made by Raper. They have developed at the center of a bacterial colony, whose edge is seen at the top of the picture. The fruiting bodies are in different stages of development: the larger ones are still building and the one at the right, with a spherical apical spore mass, is mature.

species aggregate together, in response to the same acrasin, and then the cells of the two species separate from each other at the center of the aggregate to form two separate multicellular individuals. Nevertheless, there is already evidence that there are at least eight different

acrasins among the approximately 50 known species of cellular slime molds; when all these species have been carefully investigated, additional acrasins will surely be revealed.

This means that the extent to which cellular slime molds have been able to

evolve from one chemotaxis system to another is quite astounding. It raises the fascinating question of how a signaling system can shift from one such chemical system, with its acrasin, its receptors for a specific acrasin and no doubt other associated substances, to another system. Clearly the selection pressure on slime molds to diversify and occupy new niches must be intense; even given that assumption, how are the evolutionary steps of change from a cyclic AMP chemotaxis system to a glorin chemotaxis system (or vice versa) accomplished?

I find it useful to compare slime molds in the soil to signal systems in man. Human beings have a vast array of hormones and neurotransmitters, and one assumes they arose during man's evolution from simpler animals that had far fewer signal systems. This leads to the same question as before: How did new hormones and neurotransmitters arise during the early evolution of animals? Again one could imagine that for increased control it is important to have a series of quite separate hormones and neurotransmitters, and that through natural selection some of those signaling systems gave rise to new ones, just as slime molds evolved into new species.

I am drawing a grand analogy between a mammalian body and a plot of soil and postulating for both a trend toward the increased diversification of chemical signals. It is therefore conceivable that the origins of acrasins may help us to understand the origins of animal neural and hormonal signaling systems. There is, however, a difference of staggering proportion between slime molds and mammals: the signals in animals, including human beings, produce an individual organism with extraordinarily unified accomplishments, including noble thoughts, something one can hardly expect of a shovelful of soil.

## The Author

JOHN TYLER BONNER is George M. Moffett Professor of Biology and chairman of the department of biology at Princeton University. He has three degrees from Harvard: a B.A. from Harvard College (1941) and an M.A. (1942) and a Ph.D. in biology (1947) from Harvard University. He writes: "In 1939 while an undergraduate I became interested in cellular slime molds (or social amoebae) as organisms ideally suited to experimental studies in developmental biology. It was a case of love at first sight and we have been going steady ever

since. After graduate work and a stint in the Air Corps I came to Princeton, where I remain. Besides continuing my experimental work I have written a number of books, which mostly revolve around the general theme of evolution and development from cells to societies." Among Bonner's books are *Cells and Societies* (1955) and *On Development: The Biology of Form* (1977).

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## Patterns of glucose utilization and protein synthesis in the development of *Dictyostelium discoideum*

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**Abstract.** Using autoradiography, we examined the distribution of glucose utilization with labelled deoxyglucose, and protein synthesis with labelled leucine. Glucose utilization showed an even distribution at the early stages of slug migration and then, after the appearance of distinct prestalk-prespore zones, it showed a uniformly high level in the posterior prepore cells and a uniformly low level in the anterior prestalk cells. Occasionally, an intermediate gradient, highest at the posterior end, was observed which indicated a possible intermediate stage of slug migration. In all stages of slug development, the protein synthesis showed a gradient which was highest at the posterior end. This suggests that the posterior spore-formation region involves more active metabolic activities than the anterior tip, which is responsible for the organization of the cell mass.

### Introduction

There has been a great interest over the past few years in pattern formation in the cellular slime molds. This has included an accumulation of a variety of relevant and numerous facts, both biochemical and mathematical (for reviews, see [15, 18, 19]). The basic problem can be described in the simplest terms: after an aggregation of starved cells, which already show some early signs of differentiation into either the stalk cell or spore direction (e.g., see [24, 31]), the resulting cell mass or slug shows, at some time approximately within the first 3 h of migration [5], a distinct, relatively small, anterior prestalk zone, and a larger, posterior prepore zone. The proportions of the volumes of these two areas retain a constant relation over a large range of slug sizes [4]. During the final fruiting phase (culmination), the spore and stalk cells become fully differentiated and maintain a proportionate relationship in different-size fruiting bodies [15, 32]. Finally, it is known from the early work of Raper [21] that a slug can be cut into fractions and, given sufficient time (more is needed for the anterior portions), each one will produce a normally proportioned fruiting body. Clearly, prestalk cells can convert to prepore cells and vice versa; these presumptive cells are not determined and their fates can be reversed. (For further interesting information on the process of regulation see, among others, [9, 23, 27].)

Everyone agrees that we need more facts to understand this differentiation pattern. There have been many studies

that have compared the entire prestalk zones with the prepore zones and one finds differences in the contents of mRNAs [17], in specific proteins, including various enzymes, and in total cyclic AMP. Even more useful from the point of view of pattern formation are those studies that have shown a spatial pattern of the distribution of substances along the axis of the developing cell mass. They involve the distribution of various enzymes, simple sugars, polysaccharides, calcium, ATP, cyclic AMP, and DNA synthesis. (This is work from numerous laboratories: for a review see [15].)

The observations presented here add two basic biochemical activities to the list: (1) the spatial distribution of glucose utilization as measured by the amount of accumulation of deoxyglucose; (2) the distribution of total protein synthesis within the developing cell mass. As will be evident, the patterns of distribution obtained were quite unexpected and may serve as a useful contribution towards our understanding of the basic mechanisms involved.

### Methods

Strain NC-4 of *Dictyostelium discoideum* was grown with *Escherichia coli* B/r on nutrient agar (10 g peptone, 10 g dextrose, 0.381 g Na<sub>2</sub>HPO<sub>4</sub>, 1.45 g KH<sub>2</sub>PO<sub>4</sub>, 20 g Bacto-agar, 1,000 ml distilled H<sub>2</sub>O) at 21° C. After feeding, the starved cells were washed 3 times for 5 min each by centrifugation at 75 g in 1% (10<sup>-4</sup> M) standard salt solution [1]. The cells from the pellet of the final centrifugation were sucked up in a pasteur pipette and placed in an extruded worm on a Petri dish of non-nutrient (2%) agar. The cells were placed on one side of small slips (ca. 3 × 3 mm) of millipore filter paper, and the Petri dish was placed in a closed box with holes in the side so that the emerging migrating slugs would move towards the unilateral light and onto the filter paper slips.

After about 17 h at 21° C, when many migrating slugs had emerged onto the filter paper slips, they could be picked up with forceps and placed directly (with the slips) into wells containing the labelled material. Slugs were of two classes: those that had not migrated at all and had fallen on the filter paper lying adjacent to the initial line of cells, and the slugs that had migrated at least 5 mm (which is roughly the equivalent of 5 h of migration).

Two different labelled substances were used: (1) [<sup>3</sup>H] 2-deoxy-D-glucose (37.3 Ci/mM); (2) [<sup>3</sup>H] leucine (60 Ci/mM), both from New England Nuclear, Boston, MA



02118, USA. A 20  $\mu$ l drop of either substrate was placed in a small glass well covered with a slide (in the deoxyglucose experiments only) and a Petri dish cover to avoid evaporation. The dishes were covered with black paper in order to discourage culmination. A slip of filter paper holding a slug was placed on the drop and the labelled substance was filtered through the millipore filter to the slug for 1 or 2 h. The slugs tended to round up and were subsequently placed in a light-tight box for approximately 2 h, during which time they resumed their elongate shape.

The fixation procedure for the [ $^3$ H]-deoxyglucose series was to place the slugs in acetone (electron microscopy grade, Ernest F. Fullam, Inc., Schedneckady, New York) with or without basic fuchsin overnight at 4° C [22]. The fixation procedure for the [ $^3$ H]-leucine series was with glutaraldehyde and osmium tetroxide (Polysciences Inc., Warrington, PA 18976, USA) as follows: (a) 2 ml of 1% glutaraldehyde in 25 mM of sodium cacodylate (pH 7.1) for 15 s; (b) 1 ml of 2% osmium tetroxide in 25 mM of cacodylate added for 15 min; followed by (c) 6 washes, 10 min each of 25 mM cacodylate; and (d) left overnight at 4° C in the last wash [29].

Some slugs were fixed in (a) 4% formaldehyde (pH 7.3, with 50 mM of  $\text{Na}_2\text{HPO}_4$ ) for 1 h with 1 change, followed by (b) 3 washes (10 min each) with 5 mM  $\text{Na}_2\text{HPO}_4$  and 5 mM leucine followed by (c) 5 washes (10 min each) of the  $\text{Na}_2\text{HPO}_4$  alone. Both the acetone- and the glutaraldehyde-fixed slugs were imbedded in Spurr's medium [26]. JB-4 plastic embedding medium [7] was used for the slugs fixed in formaldehyde.

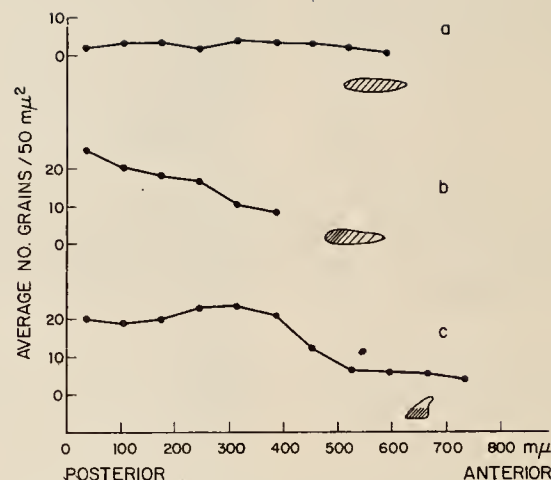
After sectioning (thickness of sections was 2–3  $\mu$ m), the slides were prepared for autoradiography and incubated for periods of time from about 2 weeks for [ $^3$ H] leucine and 10–15 weeks for [ $^3$ H] deoxyglucose. Once the emulsion was developed and the slides covered with coverslips, the grains were counted along the length of a slug under oil immersion using a grid of small squares, each 50  $\mu\text{m}^2$ . The number of grains per square was averaged for every ten squares in a row and was used for the ordinates in the figures.

## Results

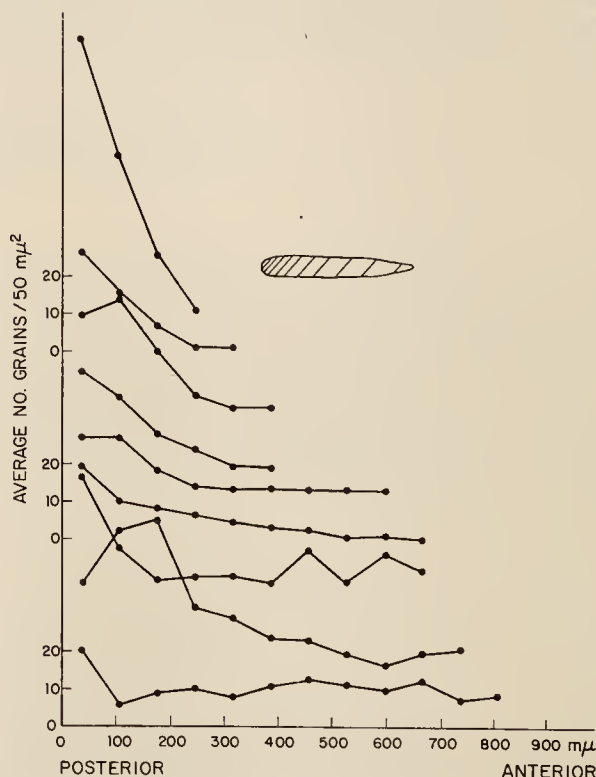
### Deoxyglucose

Measuring the metabolic activity of cells by adding labelled deoxyglucose and observing its accumulation has been widely used in neurobiology to identify physiologically active neurons [25]. Here we used it to measure the metabolic activity of a developing system.

Migrating slugs that had either not migrated at all or had migrated at least 5 mm were labelled for 2 h with [ $^3$ H] deoxyglucose. Of 22 slugs which were successfully sectioned and showed clear grains in the autoradiographs, it is obvious that each slug had achieved a different degree of maturity and that the distance it had migrated before labelling was not a reliable index of the extent of development. If one bases maturity on the appearance of the cells (i.e., the clarity of the division between prestalk and prespore zones, and, occasionally, the development of a stalk), one can see that there are three different stages of the pattern distribution of the grains from the [ $^3$ H] deoxyglucose. The first is seen in the youngest slugs which show an even distribution of grains along the whole length of the slug (Fig. 1a).



**Fig. 1a-c.** Graphs showing three different patterns of grain distribution in autoradiographs of longitudinal sections of migrating slugs indicating the accumulation of [ $^3$ H] deoxyglucose: **a** In slugs that appear to be beginning their migration. There is an even distribution of grains along the anteroposterior axis (one of six similar cases). **b** In some instances, there is a gradient of grain concentration, highest at the posterior end. These are presumed to be migrating slugs of intermediate age (one of four similar cases). **c** In mature migrating slugs, where there is a distinct prestalk and prespore zone, the grains show two even levels: high in the posterior, prespore region and low in the anterior, prestalk region (one of four similar cases)



**Fig. 2.** Graphs of migrating slugs showing the incorporation of [ $^3$ H] leucine into newly synthesized protein. Note that, in all cases, the protein synthesis is highest at the posterior end

Those slugs that clearly have formed distinct prespore and prestalk zones (some of which have an early stalk running through the zones) have a sharp division between the number of grains in the prespore zone, which is high and uniform, and the prestalk zone, which is low and also uniform (Fig. 1c). In a number of instances, there was a distinct gradient which was highest at the posterior end and lowest at the anterior end. The appearance of the slugs suggested that these were slugs of intermediate maturity (Fig. 1b).



## Leucine

Total protein synthesis can easily be measured by adding a labelled amino acid that will become incorporated into newly formed protein. When [ $^3\text{H}$ ] leucine was added, it was invariably true that the highest amount was incorporated into the posterior end of the slug and there was a gradient of declining intensity in the anterior direction. This was carefully measured in nine slugs (Fig. 2) and observed in others, including some in which a stalk was beginning to form.

## Discussion

There has been a general assumption, as a direct result of the influence of Child [8], that the tip of a slug, which has organizing properties or "dominance" (in the sense used by Child [11, 21]), will also be the region of highest metabolic activity. It is also assumed that glucose utilization is involved in energy conversion in the cellular slime molds. From the results presented here, it is obvious that the cellular slime-mold cell mass does not achieve its apical dominance by establishing a gradient of glucose utilization.

However, Child based his generalization largely on oxygen consumption, and we know from the work of Sternfeld and David [28] that gradients of oxygen are capable of orienting the cells towards high concentrations and, therefore, determining the polarity, including the region where the tip will form. The role of oxygen utilization may be different from that of glucose utilization: a matter worth further investigation.

It is important to note that the pattern of glucose metabolism we report here is identical to the pattern found for mitochondrial enzymes such as succinic dehydrogenase and cytochrome oxidase [30]. Also, it should be noted that non-graded differences in activity between prestalk and prespore cells have been found for vital staining [3], alkaline phosphatase [6, 12, 13], calcium [16], bound cyclic AMP [20], and fucose incorporation [10]. Finally, it may be relevant that Leach et al. [14] showed that the addition of glucose to vegetative amoebae caused them to sort out preferentially into the posterior, prespore zone.

The distribution of protein synthesis in the slug is again an unexpected result: we had assumed that, if there was a gradient, it would be highest at the anterior end; however, the reverse is true and seems to be maintained throughout the development of the slug and into early culmination. Our results could, of course, be due to a distortion and not represent the true distribution of protein synthesis. If there is a steep gradient of the size of the unlabelled amino acid pool, then the percentage of labelled leucine would automatically be large where the pool was small. There is no evidence that there is such a pool gradient, but the possibility cannot be ignored.

While there may be many substances that are produced in a graded fashion, the only one that has been demonstrated is the secretion of cyclic AMP [2]. It is of special interest that recently S.L. Barclay (personal communication) has evidence that cyclic AMP inhibits protein synthesis, which might explain why the cyclic-AMP secretion gradient is the reverse of the protein synthesis gradient.

These results on the pattern of metabolism and protein synthesis within the slug suggest that we have much to learn before we will achieve a complete understanding of the mo-

lecular basis of developmental patterns in the cellular slime molds.

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## pH affects fruiting and slug orientation in *Dictyostelium discoideum*

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### SUMMARY

We have demonstrated two interesting facts about the transition from the migration stage to the final fruiting stage of *Dictyostelium discoideum*. One is that fruiting is favoured on acid substrata, and secondly, migrating slugs tend to migrate towards the acid side of a pH gradient. The suggestion is offered that these results can be interpreted in terms of the effects of  $\text{NH}_3$ . It appears to be an additional mechanism (besides phototaxis and thermotaxis) to assure that the final fruiting takes place in a favourable environment.

### INTRODUCTION

There has been a concern for some time with the question of what factors influence migrating slugs of *Dictyostelium discoideum* to turn into fruiting bodies. Raper (1940) reported that light, a rise in temperature, and a decrease in humidity all favoured early fruiting. Later we suggested that humidity played an especially significant role (Bonner & Shaw, 1957) although this interpretation of those experiments is doubtful in the light of recent work. Next, Newell, Telser & Sussman (1969) reported that overhead light stimulated fruiting and that there was a factor given off by the slugs that enhanced migration. Schindler & Sussman (1977) discovered that this chemical stimulus for migration was  $\text{NH}_3$  and that the absence of  $\text{NH}_3$  encouraged fruiting. More recently, we examined the effect of overhead light and found that the light lifted the slug off the agar by phototaxis, and that this positional change of the slug promoted fruiting (Bonner *et al.* 1982).

Here we present experiments which show that the pH of the substratum not only affects the transition to fruiting, but migrating slugs are oriented by pH gradients. Earlier, Raper (1939) reported that if drops of HCl were placed near colonies of amoebae, the more HCl in the drop, the greater the number of fruiting bodies. Furthermore, he observed that the slugs migrated towards acid regions in the Petri dish. We have tried, unsuccessfully over the years to repeat this experiment, but never could exactly duplicate the required conditions. Now, using a totally different method, we have confirmed Raper's original observations. Furthermore, we suggest an hypothesis that brings together the role of light and  $\text{NH}_3$  in the stimulation of fruiting *D. discoideum*.

*Keywords:* pH, gradient, *Dictyostelium*, slug orientation, fruiting.



## MATERIALS AND METHODS

*Dictyostelium discoideum* (NC-4) was grown with *Escherichia coli* (B/r) at 21 °C on nutrient agar. The amoebae were washed by centrifugation and concentrated in masses on non-nutrient agar (see Bonner *et al.* 1982, for details) to obtain large migrating slugs. The first set of experiments were run at 21 °C, and the pH gradient experiments were done at 12 °C, the latter temperature being especially favourable for prolonged slug migration.

*Fruiting body formation and pH*

A series of five experiments were run in which developing amoebae were composed on sets of Petri plates containing agar at two different pH concentrations. Half of the plates (at both pH's) were placed in the dark, while the other half were placed in a chamber with a source of light under the agar to attract the tips toward the agar surface so that the slugs would tend to hug the agar (see Bonner *et al.* 1982, for details). At different time intervals (plating the amoebae = time 0), the plates were removed and scored for percent fruiting (*vs.* percent migrating slugs). This was done by placing the plates over a grid divided into 0.5 mm squares and they were examined under a dissecting microscope at a magnification of  $\times 25$ . The number of slugs and fruiting bodies for a series of grid squares were scored involving a total of 100–400 pseudoplasmodia for each experimental condition. In all but one case, there were duplicate plates which were scored separately.

In the first two experiments, NaHP0<sub>4</sub>/KH<sub>2</sub>P0<sub>4</sub> buffer was used at 10<sup>-2</sup> M. The initial pH in one set of plates was that of the buffer mixture: the more alkaline set of plates was produced by adding different amounts of 1N-NaOH. Agar was then added to the buffer, autoclaved and poured into Petri plates (15 × 85 mm, plastic). The pH was determined before adding the agar, after the plates

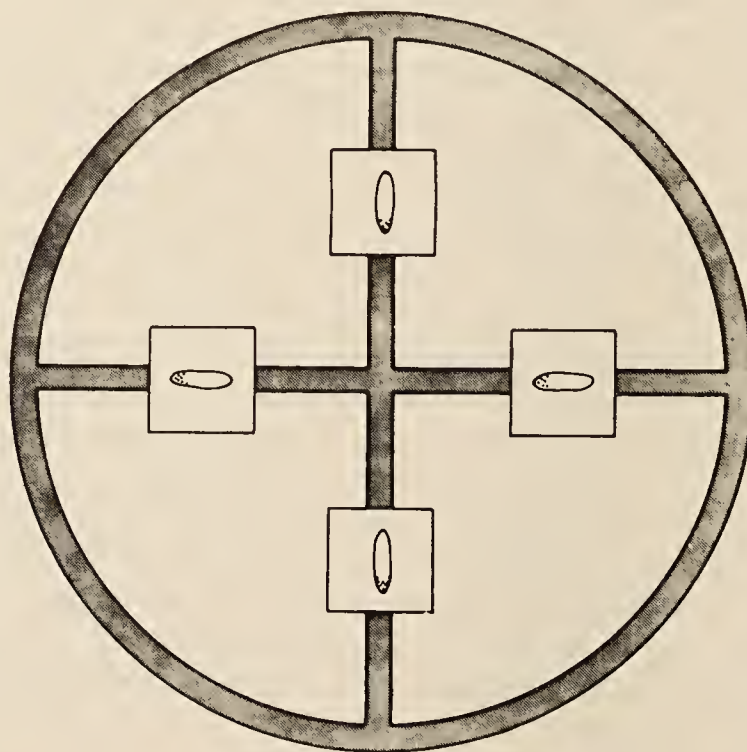


Fig. 1 A diagram (not drawn to scale) showing the placement of the slugs in the compartmentalized Petri dishes.

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had cooled, and after the experiment was over; in each case the values held reasonably steady, even when the pH was at the extreme range of the buffer. In the next three experiments, a  $\text{NaHP0}_4$ /citric acid (Sorensen's) buffer was used at  $16.7 \times 10^{-3}\text{M}$ . Care was taken to see that the total concentration of solutes in the agar did not vary significantly since it has been stated previously that an increase in the solutes results in a higher percent fruiting of bodies formed in a given time interval (Slifkin & Bonner, 1952). In the first two experiments, the paired pH's were 6.3–8.9, and 6.0–12.0. In the next three, they were replicates at pH 6.7–8.0.

The final experiments were run in Felsen-type Petri dishes in which the agar is in four separate quadrants or compartments (Falcon X plastic Petri dishes— $15 \div 100\text{ mm}$ ). Agar of one pH is put in two opposing quadrants, and the agar of another pH is put in the intervening quadrants. Each quadrant is separated from its neighbour by a plastic ridge. As before, slugs were formed from centrifuge-washed amoebae, placed on 2% agar. Slugs that had separated out from their neighbours were removed by cutting the 2% agar under them in a block approximately 15 mm square. These were then placed on the ridges of the compartments with the orientation shown in Fig. 1. In this way, the agar block straddled the plastic ridge and touched agar of two different pH's on each side. The experiments were run at  $12^\circ\text{C}$  in the dark.

Three different buffer systems were used: MES for pH 5.5, 6.0 and 6.5; HEPES for pH 7.0, 7.5 and 8.0; and TRIS for pH 5.5, 6.0, 7.5 and 8.0, all at  $15 \times 10^{-3}\text{M}$ . Controls were run for all these buffers at the stated pH's in which all four compartments contained the same buffer. Again the pH's were tested before and after the experiment, and the deviation was found to be negligible. After 2 h and 4 h, the slugs were scored for their direction of migration.

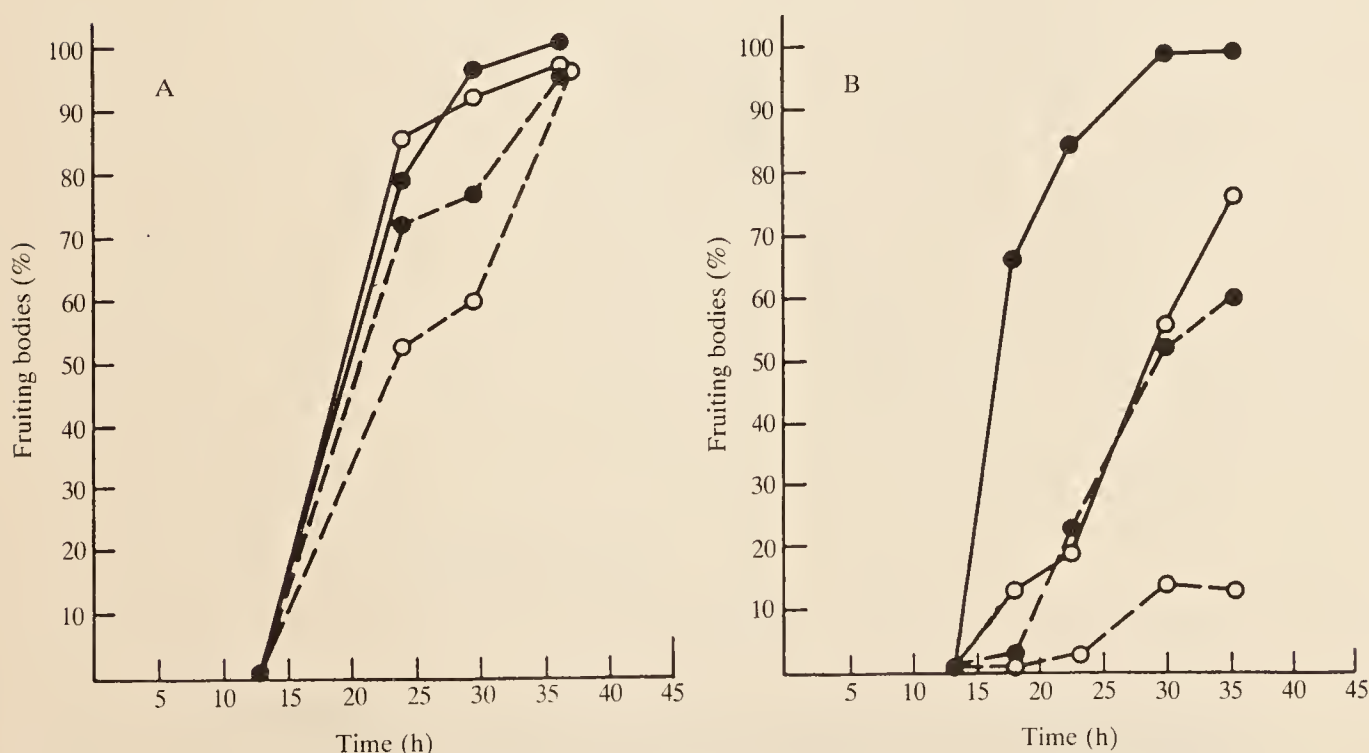


Fig. 2 The increase in the percentage of fruiting bodies over time under different conditions of light and pH. Open circles = light below; solid circles = dark. (A) pH 6.7 (solid line) compared with pH 8.0 (dashed line). (B) pH 6.0 (solid line) compared with pH 12 (dashed line). Given enough time all the slugs turned into normal fruiting bodies under all conditions.



## RESULTS

*The effect of pH on the rate of fruiting body formation*

In all the experiments, both in the dark and with the light below, the fruiting bodies formed more rapidly in the more acid or low pH plates. This can be clearly seen in one of the three pH 6.7–8.0 experiments (Fig. 2A) and is very striking in the pH 6.0–12.0 experiment (Fig. 2B).

If the time to achieve 50% fruiting body formation is averaged in the four experiments in which the pH difference is approximately 2.0 units, in the higher pH plates it takes 2 h longer in total darkness, and roughly 4 h longer in the plates with light below. The effect is even more obvious in the fifth experiment where the pH difference is 6 units (Fig. 2B). Therefore, a combination of high pH and light below

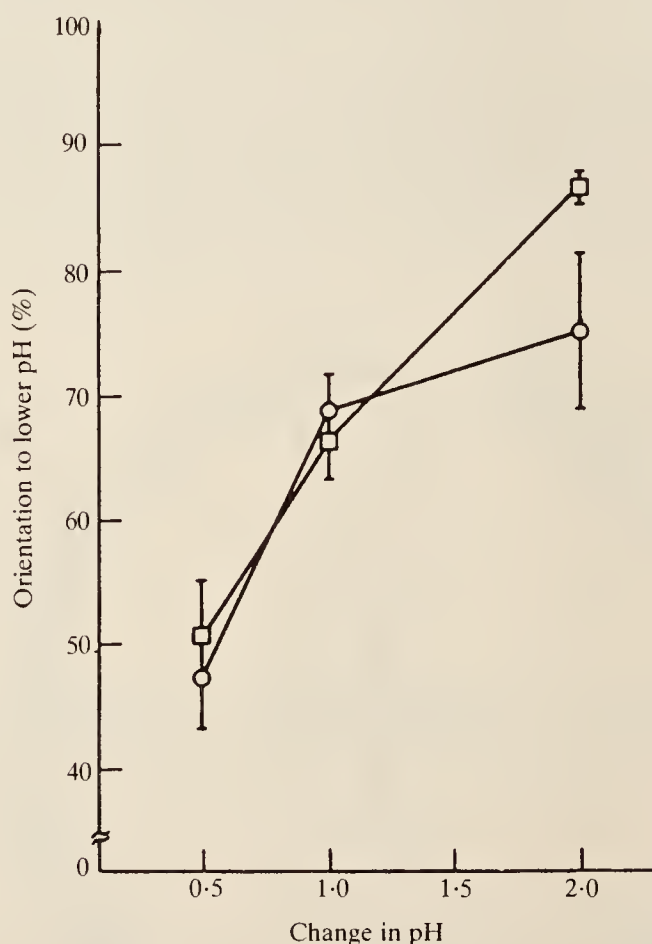


Fig. 3 The percent of slugs orienting towards the more acid side of a gradient plotted against the steepness of the pH gradient. Circles = 2 h; squares = 4 h recordings. (Data from seven experiments involving 448 slugs. The slugs that remained unoriented or twinned were not included.) For the pH differences of 0.5 and 1.0 all three buffers, MES, HEPES, and TRIS gave essentially the same result and were therefore combined. For a pH difference of 2.0 TRIS was used at pH 5.5–7.5 and 6.0–8.0, again with similar results and combined.

(which causes the slug tips to point into the agar surface) is especially effective in prolonging migration.

*Evidence for migration slug chemotaxis in pH gradients*

We have a total of seven experiments in which migrating slugs were placed in different pH gradients. When all the data from these experiments were combined, it was obvious that the steeper the pH gradient, the greater the orientation towards the acid side (Fig. 3). If the difference in pH's in the quadrants was 2.0 units, then by 4 h, 87 % of the slugs were oriented towards the lower pH. This was true for both ends of the pH scale tested. Orientation in all the control plates was random (in 11 experiments, involving 558 slugs).

DISCUSSION

In a previous study (Bonner *et al.* 1982) it was shown that the reason overhead light induces fruiting in migrating slugs, as Newell *et al.* (1969) had discovered, was that the light lifted the tip of the slug from the agar surface by phototaxis and this lifting process led to fruiting. In view of the results described here, we can ask what might be the molecular stimulus that causes aerial tips to lead to the final differentiation of spores and stalk cells, and the formation of a fruiting body.

Initially we postulated that it might have something to do with surface tension within the water film and if the slug tip is in the film, migration continues. This hypothesis was put forward on the presumption that the whole reason for fruiting is to break through the surface film and push the spore mass up into the air. Using a non-toxic detergent (Tween 20) we were able to lower the surface tension of the water film significantly, but this in no way affected the rate of fruiting (data not shown).

Our results presented here showing that low pH favours fruiting leads us to suggest that the overhead light effect on fruiting could best be explained in terms of ammonia relations. Schindler & Sussman (1977) and Sussman & Schindler (1978) demonstrated that an increase in ammonia caused a prolongation of migration. If we ask what effect pH has on ammonia, the higher the pH the greater the  $\text{NH}_3/\text{NH}_4^+$  ratio. Our results in which alkaline agar favours migration and acid agar favours fruiting are totally consistent with the Schindler and Sussman observations; relatively more  $\text{NH}_3$  favours migration.

Now we must account for the fact that when the light is below, as compared to total darkness, migration is prolonged even further. It is possible to imagine two ways in which  $\text{NH}_3$  influences fruiting: by the overall amount of  $\text{NH}_3$  due to the effect of pH, and by the amount of  $\text{NH}_3$  lost from the surface of the slug by diffusion. Slugs in the dark will be oriented at random, many of them rising into the air. Those slugs surrounded by air will lose  $\text{NH}_3$  far more rapidly than those which cling to the water film on the surface of the agar. If this hypothesis is correct, then the reason overhead light promotes fruiting is that it allows  $\text{NH}_3$  to diffuse rapidly away from the free slugs that rise from the agar surface by phototaxis.



Let us consider the intriguing fact that slugs seem to migrate towards a low pH environment that favours fruiting. This chemotactic response of whole slugs is weak by comparison to their phototactic and thermotactic response, and, as one can see from Fig. 3, a relatively steep pH gradient is needed in order to produce a significant effect. If the pH difference between the two quadrants of the Petri dish is 2.0 then one can assume that over a distance of about 3 mm, the gradient would involve a 100-fold difference in hydrogen ions. We have only tried fair-sized slugs, so assume that the width of the slug is 0.3 mm, then this will produce a difference of 10-fold in  $H^+$  between the sides of a slugs. It is quite possible that it is not the  $H^+$  gradient that is significant, but the gradient of  $NH_3$ . This raises the further interesting possibility that  $NH_3$  might be the repellant gas involved in the orientation of rising pseudoplasmodia described previously (Bonner & Dodd, 1962).

Finally we can ask whether or not this pH chemotaxis might have some ecological significance. There is a common thread in all migrating slug taxes: they bring the slugs from regions of the soil where fruiting is less desirable to where it would be more so. And the presumed reason for this is that positions near the surface of soil are more effective for spore dispersal, while deeper regions are more suited to the earlier feeding stage. This would explain why the slugs go towards light. It would also explain the extraordinary sensitivity to temperature gradients (Bonner, Clarke, Neeley & Slifkin, 1950; Poff & Skokut, 1977), especially since recently Whitaker & Poff (1980) have shown that at cool temperatures migrating slugs are negatively thermotactic, while at warmer temperatures they are positively chemotactic, and the range of these two effects is determined by the temperature at which the amoebae grow. This could be explained by the fact that at night the soil surface would be colder than below (hence negative thermotaxis of the slugs if they want to move towards the surface), while in the daytime the reverse is true, and again this would lead to the slugs going upward. We can now add to this list of speculations the possibility that migrating slugs can move away from regions high in  $NH_3$  which would be characteristic of deep chambers in the soil laden with bacterial food, to regions relatively free of  $NH_3$ , such as the more aerated soil surface. If there is any truth to these ideas, it is remarkable that so many different mechanisms evolved to insure spore masses being placed optimally for dispersal.

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SUCCESSIVE ASEXUAL LIFE CYCLES OF STARVED  
AMOEBAE IN THE CELLULAR SLIME MOULD,  
*Dictyostelium mucoroides* VAR.  
*STOLONIFERUM*

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SUMMARY

Dense masses of spores of *Dictyostelium mucoroides* var. *stoloniferum* have the ability to germinate and aggregate rapidly in the absence of food. This is made possible by the presence of a dominant, self-produced spore germination activator. The germination–aggregation cycle can be repeated in as many as six successive generations. In each generation the spore size is reduced so that ultimately they are only a fraction of the size of those produced by the parental, bacteria-fed amoebae.

INTRODUCTION

In their original description of the cellular slime mould *Dictyostelium mucoroides* var. *stoloniferum* (henceforth referred to as *stoloniferum*) Cavender & Raper (1968) pointed out the unusual feature of this particular form. When one of the long stalks falls over on the substratum, the spores germinate rapidly and if there is no nutrient present in the form of bacteria the amoebae will aggregate and develop into a diminutive fruiting body (Fig. 1). While it has been known for some time that many species can be forced to have a second generation without feeding, including *D. discoideum*, *stoloniferum* has the advantage of doing this normally.

Because of these unique properties, there are certain kinds of experiments that can be done with *stoloniferum* that are difficult or impossible to do with the other species. Here we concentrate on two specific aspects.

One is the germination properties of *stoloniferum*. In most species, the denser the spore inoculum, the lower the percentage germination (Russell & Bonner, 1960; review by Cotter, 1975), while, as we will see, the reverse is true for *stoloniferum*.

The other is the fact that starved amoebae will aggregate and form fruiting bodies with smaller cells. We have been able to do this over a series of successive generations and produce very small amoebae. Furthermore, it is possible to mix the cells of different generations (i.e. different sizes) and see whether they sort out in the cell mass during subsequent development.

Key words: *Dictyostelium*, *stoloniferum*, starvation.

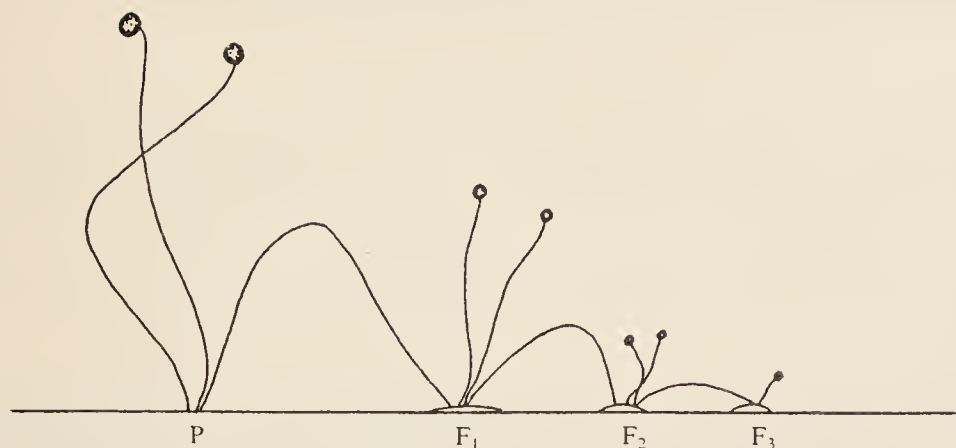


Fig. 1. A diagrammatic representation of four successive generations of *stoloniferum*. If a fruiting body falls over, the spores germinate and produce a new set of smaller fruiting bodies.

Therefore, by taking advantage of the special properties of *stoloniferum*, we have been able to gain new insights into the process of spore germination, cell sorting and the effects of starvation on development.

#### MATERIALS AND METHODS

These experiments were done on *Dictyostelium mucoroides* var. *stoloniferum*, strain number FO-II-1 kindly supplied by Dr K. B. Raper. They were grown at 22 °C in mixed culture with *Escherichia coli* B/r on nutrient agar (10 g peptone, 10 g dextrose, 0.381 g Na<sub>2</sub>HPO<sub>4</sub>, 1.45 g KH<sub>2</sub>PO<sub>4</sub>, 20 g Difco agar in 1000 ml distilled water).

In the starvation experiments spores were removed from sterile, aerial sori and placed on 2 % non-nutrient agar containing 0.25 g/l streptomycin sulphate to prevent bacterial growth. (Comparisons of the sizes of spores developed on agar with and without the streptomycin showed no difference.) All the cultures were incubated at 22 °C in continuous, diffused light.

The procedure for serial transfer involved first picking up spores from aerial sori in densely inoculated stock culture plates grown on nutrient agar with a small platinum loop and placing them on streptomycin agar. Twenty-five such loopfuls were placed in small heaps roughly 4 mm apart in a solid circle approximately 2 cm in diameter. At least 25 Petri plates were inoculated in this fashion for the first starved generation, and this was repeated in the subsequent generations, each time with a decreasing number of loopfuls.

The lengths (and in one experiment the widths) of the spores from each generation were measured directly with an ocular micrometer using an oil-immersion, phase-contrast microscope ( $\times 1600$ ).

Spores from the original bacteria-fed amoebae are referred to as the P generation, and the subsequent starved generations are labelled F<sub>1</sub>, F<sub>2</sub>, F<sub>3</sub>, etc.

For the scanning electron micrographs, the following technique was used. Fruiting bodies of given generations were touched with coverslips and spread with one drop of standard saline (Bonner, 1947). A small volume of concentrated polystyrene latex microspheres (Duke Scientific) of 1.10 ( $\pm 0.10$ )  $\mu\text{m}$  diameter was added and allowed to settle for half an hour. The coverslips were drained, dipped in water and then each placed in 1 ml of 1 % glutaraldehyde in 25 mM-cacodylate buffer (pH 7.1). Fifteen seconds later, 0.5 ml of 2 % OsO<sub>4</sub> in cacodylate buffer was added. Spores were incubated for 20–30 min at room temperature, rinsed six times for 10 min each in cold 25 mM-cacodylate buffer and dehydrated in a cold ethanol series. The coverslips were finally processed in a critical-point dryer, then gold coated for scanning electron microscope (SEM) viewing and photographed at  $\times 2000$  magnification.



For the time-lapse films, we used a Panasonic Time Lapse Videotape Recorder (NV-8050) set at 120 h/24 h. This was attached to an inverted Zeiss microscope and the area of the field was  $19.6 \mu\text{m}^2$ . (We thank Dr D. Ready for the use of this equipment.)

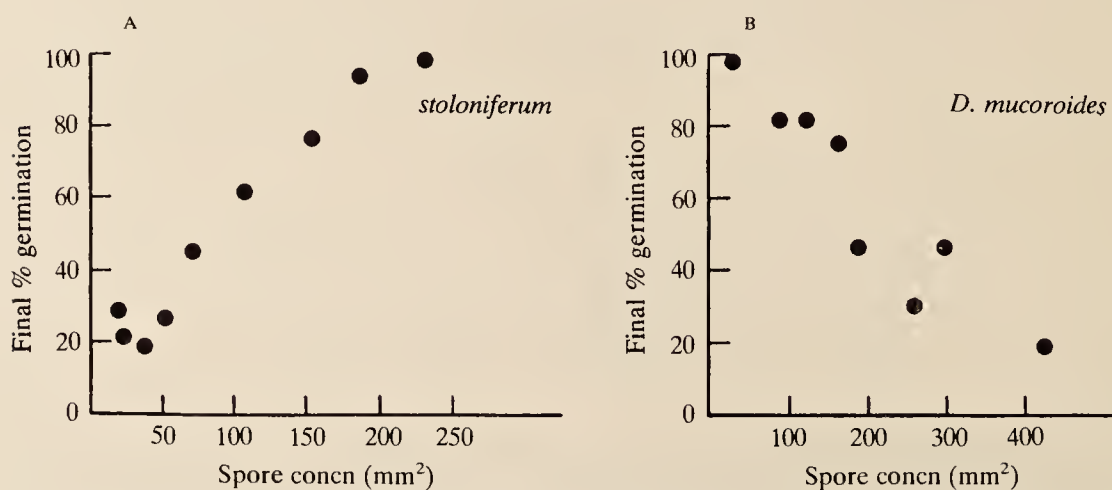


Fig. 2. A comparison of the effects of spore density on the percentage germination for *stoloniferum* (A) with normal *D. mucoroides* (B); (B is from Russell & Bonner, 1960).

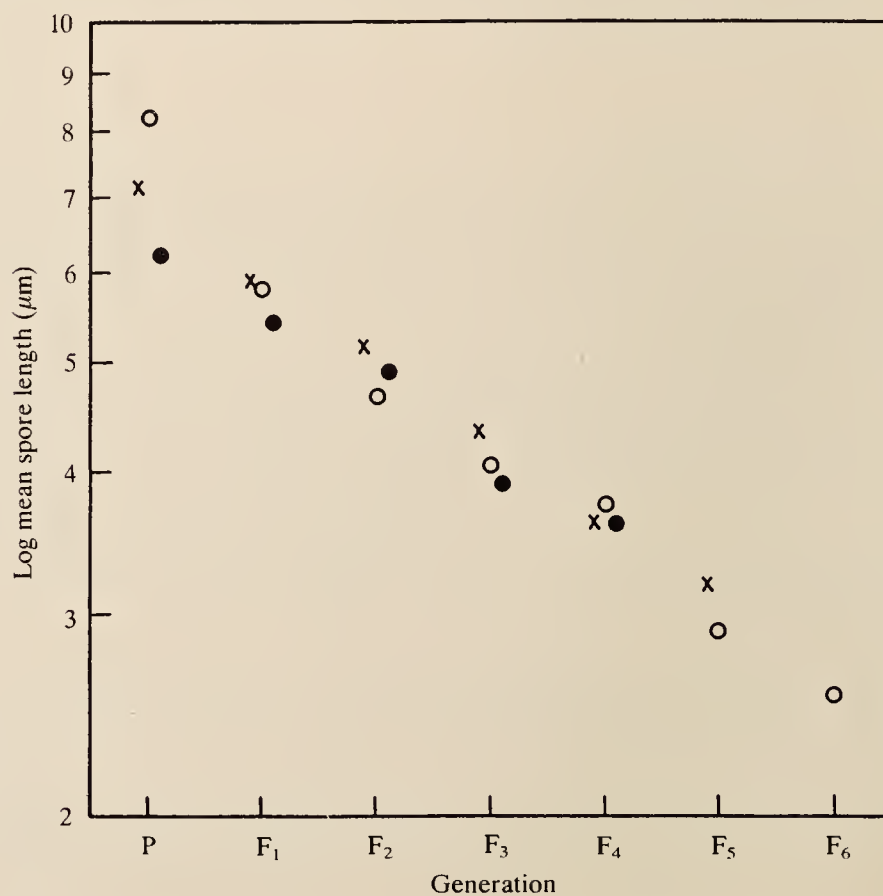


Fig. 3. The logarithm of the spore length as it declines over successive generations of starvation. The points are for the means of three separate experiments. (The  $\times$  points are means of 150 spores; the other points are for 100 spores each).



## RESULTS

*The effects of cell density on spore germination*

If different concentrations of spores are spread on the surface of non-nutrient (2 %) agar, using exactly the same methods as used previously (Russell & Bonner, 1960), *stoloniferum* behaves quite differently from a standard *D. mucoroides* strain. Instead of decreasing the percentage germination with increased concentration of spores, high *stoloniferum* spore density favours germination. The earlier results with *D. mucoroides*

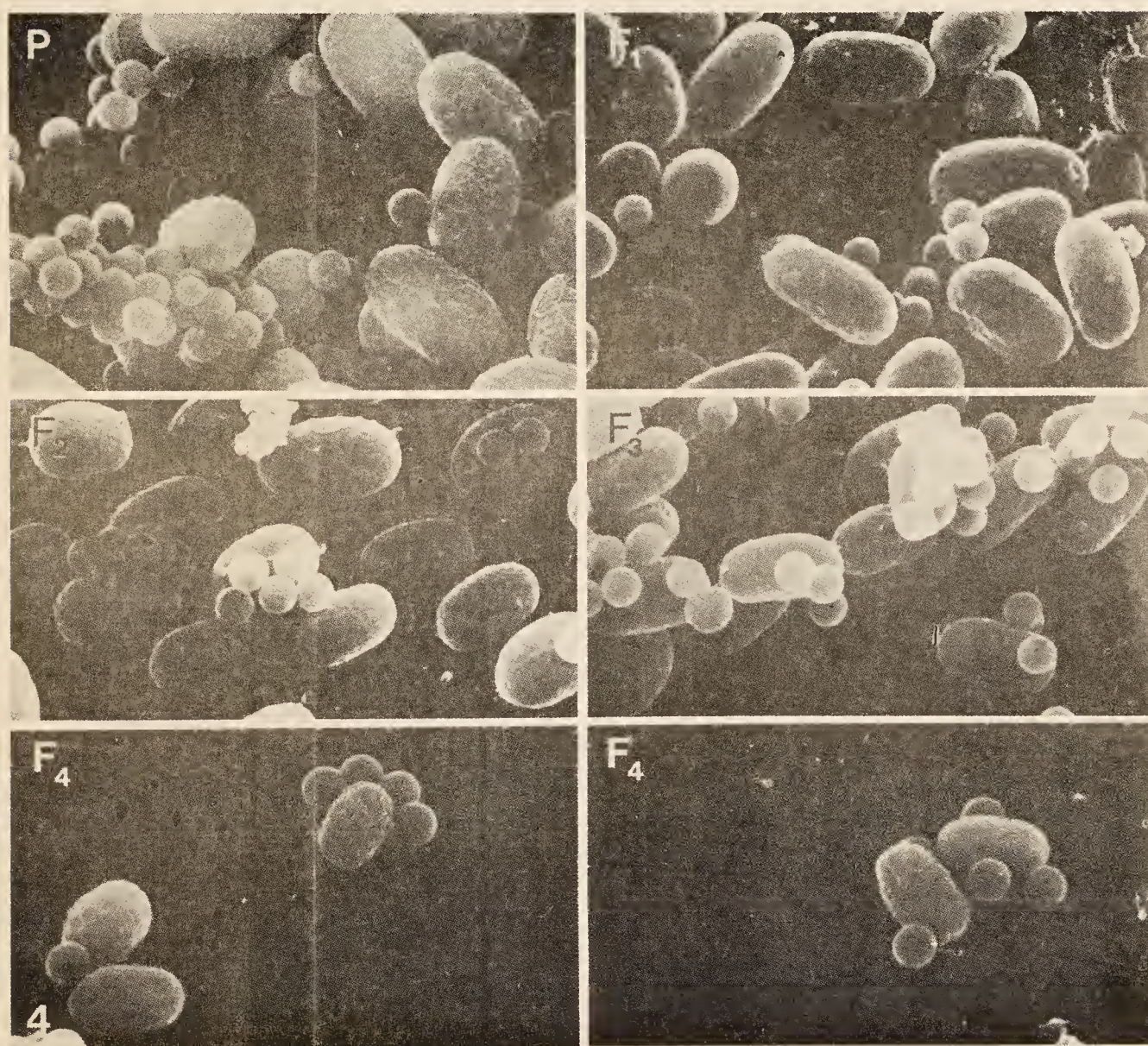


Fig. 4. Scanning electron micrographs of the spores of *stoloniferum* from the parental to the  $F_4$  generation mixed with  $1.10\ \mu\text{m}$  latex beads used for a size reference. (The beads are spherical; the spores ellipsoidal). All photographs are at the same magnification:  $\times 4545$ .



and the results obtained here with *stoloniferum* are compared in Fig. 2, where this inverse relationship can be clearly seen.

*Successive generations of life cycles without any addition of food*

Each successive generation of starvation produces a reduction in the size of the spores. This was shown in three separate experiments, which are plotted together in Fig. 3. In the most successful one, it was possible to go to the F<sub>6</sub> generation, and one F<sub>7</sub> fruiting body was obtained, but unfortunately it was so small we were unable to recover its spores for measurement.

As the spore sizes decrease they become relatively more spherical. This can be seen in the SEM micrographs comparing the P with the F<sub>4</sub> generation (Fig. 4). In one experiment, spore widths were also measured and the volume was estimated by considering the spore to be a prolate spheroid (volume =  $\frac{4}{3} \pi ab^2$ , where  $a = \frac{1}{2}$  length,  $b = \frac{1}{2}$  width). If the volumes are plotted, in this case to the F<sub>4</sub> generation, it can be estimated by extrapolation that by the F<sub>6</sub> generation the volume would be about 12 % of the P generation (Fig. 5).

Using time-lapse video tape recording of germinating and aggregating starved amoebae, we found no evidence of cell division or cannibalism. We were also interested to know if the minute F<sub>6</sub> cells would be able to engulf bacteria. When *E. coli* B/r were

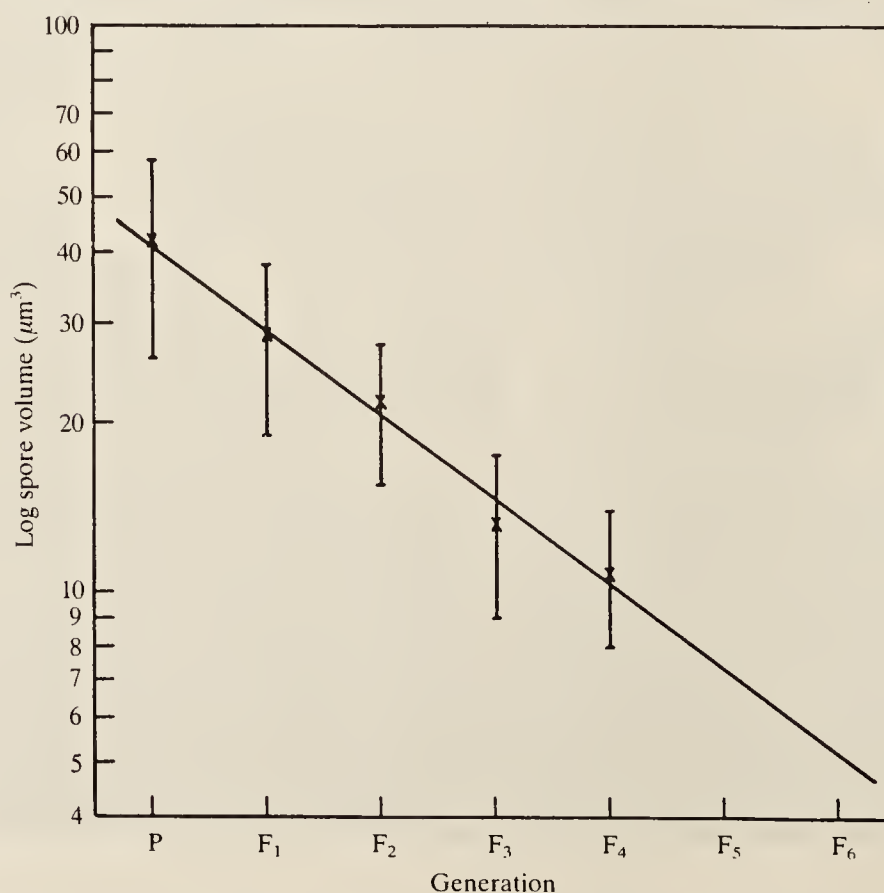


Fig. 5. The logarithm of spore volume showing the decline over successive generations of starvation. The means are of 100 spores each and the bars are the standard deviations.

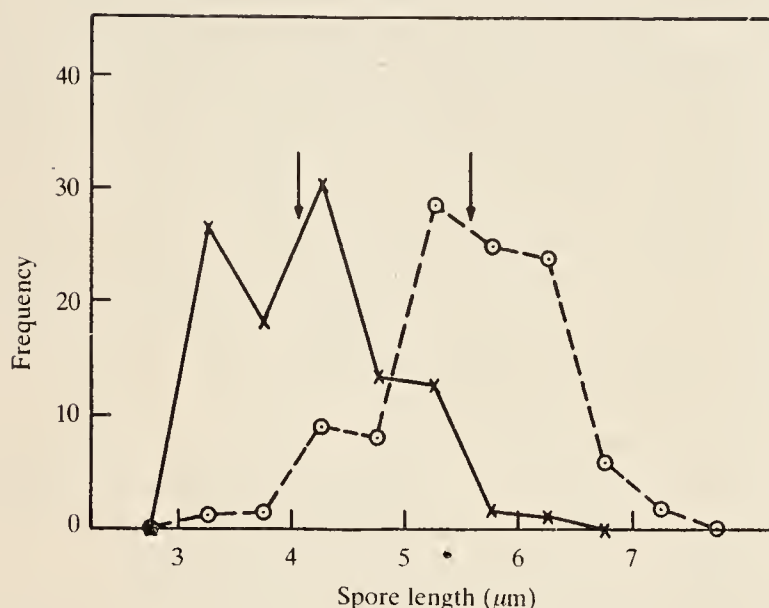


Fig. 6. A comparison of spore lengths of 500 spores from fruiting bodies derived from posterior fractions (×—×) of sorogens and 500 spores derived from anterior fractions (○---○). These sorogens are a mixture of equal quantities of large P cells and smaller cells from the F<sub>2</sub> generation.

added to such cells, they ate the bacteria and grew rapidly, producing fruiting bodies whose spore size was identical to that of the parental spores.

#### *Sorting out of mixtures of cells from different generations*

Since the cells of different generations differ so significantly in size, they can be identified by their size. Spores from two generations were mixed (we tried both P with F<sub>1</sub> and P with F<sub>2</sub>) and allowed to aggregate. As the sorogens first arose from the agar on short stalks, they were pressed down and bisected with a fine glass needle so that the anterior prestalk zone and the posterior prespore zone were separated. Both fragments were allowed to fruit and the size (length) distribution of the spores from each of the two portions was examined. The spores from the anterior fragments were significantly longer than those from the posterior fragments (Fig. 5). The larger cells from the P generation sorted out to the anterior prestalk region of the slug, while the smaller, starved cells sorted out to the prespore region.

#### DISCUSSION

*D. mucoroides* var. *stoloniferum* differs significantly from other known slime moulds in that germination is favoured rather than hindered in dense spore populations, and the resulting amoebae can go through a succession of generations or asexual life cycles without food. The resulting amoebae are exceedingly small, and the size difference between generations is appreciable. Let us now examine the implications of each of these findings.



The original discovery of Russell & Bonner (1960) followed by the work of others led to the clear demonstration that cellular slime mould spores produced an inhibitor of germination and the inhibitor itself was chemically identified by Abe *et al.* (1976). Quite independently, Dahlberg & Cotter (1978) in *D. discoideum* and Coco & Kornfeld (1978) in *D. purpureum* provided evidence that, in addition to the inhibitor, spores also produced a germination activator. Its role is not clear, but normally the effect of the germination inhibitor overshadows that of the activator. We have argued that the inhibitor is a means of increasing the dispersal power of the amoebae, for if spores land in a patch of bacteria, then a single germinating amoeba is sufficient to start a new generation and the remaining germination-inhibited spores have a chance of being transported to another patch of food. Also it is assumed that the inhibitor prevents germination of the spores in the sorus.

We must assume that the self-produced activator in *stoloniferum* plays the dominant role and it is not known if there is any production of germination inhibitor at all. Clearly this variety of slime mould has a different dispersal strategy, which involves producing a new, peripheral set of fruiting bodies if food is absent.

The enormous decrease in size over successive generations raises two questions of potential interest. One is the cytological effects of the decrease. The whole cell of an  $F_5$  is approximately the size of the nucleus of the parental generation. How do the cytoplasmic organelles and the structure of the nucleus itself become modified in this great size change?

Another aspect of interest is the use of these cells of different sizes in studying pattern formation in the cellular slime moulds. As was shown here, the cells of different sizes sort out so that the smaller, more starved cells assume a position posterior to the larger cells in the rising sorogen. It will be interesting to see what are the properties of the large *versus* small starved cells that lead to their sorting out.

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# Ammonia orients cell masses and speeds up aggregating cells of slime moulds

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We showed some years ago that the rising cell masses of cellular slime moulds repel one another, and that this is achieved by a gas given off from the cell masses<sup>1</sup>. It is now clear that this gas is ammonia. We also have evidence that NH<sub>3</sub> tends to speed up the movement of cells in aggregating streams which would account for its ability to repel; more NH<sub>3</sub> on one side of a cell mass would cause a speed-up of the cells on that side, thus making the mass veer to one side.

Cellular slime moulds feed and aggregate in a thin film of water and come together in a cell mass or migrating slug (either with or without a stalk, depending on the species) that moves towards a region favourable for fruiting. Ultimately the migrating slug points upward into the air to form a fruiting body consisting of a terminal spore mass (some species are branched) at the top of a thin, delicate stalk. It is known that during the multicellular stages there may be orientation towards light or towards heat<sup>2–4</sup> and that the slugs will migrate away from more basic regions<sup>5,6</sup>. In a previous study we were able to show that when the fruiting body (or sorogen, as it is known in its early stages) rises into the air it is guided by a volatile substance that is produced by the sorogen itself<sup>1</sup>. As a result, if two sorogens arise close together they lean away from one another; or if one rises near an agar cliff, it will lean away from the cliff. The best evidence that this was due to a gas was obtained by placing a piece of activated charcoal near a sorogen. It would move towards the charcoal, showing that the repellent is adsorbed by the charcoal. At that time we were unable to identify the gas, even though we knew from unpublished work of J. Lonski in our laboratory using gas chromatography, that slime moulds give off CO<sub>2</sub>, NH<sub>3</sub>, ethane, ethanol, ethylene, acetaldehyde and, no doubt, other volatile substances.

In order to test these chemicals we used small, tightly sealed plastic Petri dishes and drilled a hole 0.35 or 0.4 mm in diameter through the middle of the covers (Fig. 1). An agar block with the primordium of a fruiting body of *Dictyostelium discoideum* (NC-4) was placed below the hole so that the rising sorogen would come out parallel, or slightly away from the undersurface of the lid. This was precisely the result of all control experiments with lids lacking the hole. The small dish was closed (with moist filter paper inside) and placed in a large desiccator which

contained the gas to be tested and water to keep the humidity as high as possible, matching the humidity in the small dish. The desiccator was then placed in the dark at 23 °C. Orientation of a sorogen was already evident after an hour, which is approximately the time during which a small, rapidly diffusing molecule can maintain a gradient across the hole under such conditions.

With water vapour alone as a control, 5% CO<sub>2</sub>, 1% ethylene, 5–15% ethane, or 5% ethanol were allowed to come to equilibrium in the desiccator which contained 800 ml of water in the bottom. In these experiments the sorogen of *D. discoideum* tended to orient towards the hole, presumably because its own repellent could escape through the hole, and it was not affected by the added gasses (Table 1, Fig. 1a). The result was dramatically different with 0.027 mm Hg (0.0036%) of NH<sub>3</sub>, for the sorogens oriented directly away from the holes (Fig. 1b). Clearly NH<sub>3</sub> was a repellent, and it is well known that sorogens give off low concentrations of NH<sub>3</sub> (refs 7–9). The other gases were inactive even at concentrations much above any reasonable estimate of their physiological range.

How does NH<sub>3</sub> orient the sorogen? It has been assumed that the amoebae on one side of the sorogen move more rapidly than on the other, an hypothesis we proceeded to test. Aggregating amoebae of *D. discoideum* on 2% non-nutrient agar in an inverted Petri dish were exposed to mixtures of NaOH and NH<sub>4</sub>Cl placed in a small dish inside the cover of the Petri dish. In this way it was possible to test the effect of NH<sub>3</sub> on the rates of movement of amoebae at different stages of aggregation. These rates were measured by using a Panasonic video camera (WV-1850) with time-lapse (AG-6010). We were able to show

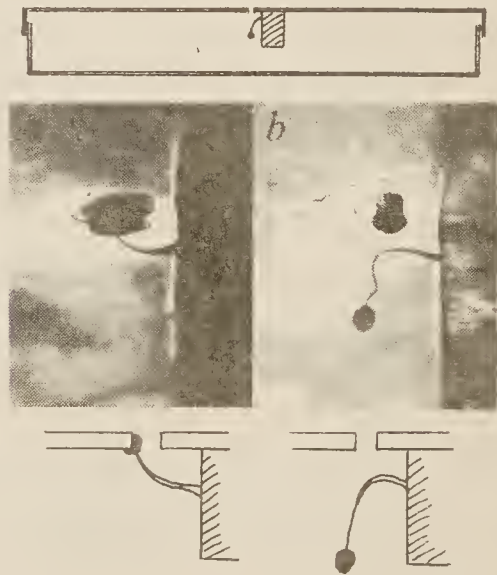


Fig. 1 The top diagram represents a section through a small plastic Petri dish (Falcon No. 1007, 60 × 15 mm) showing the position of the agar block with the slime mould sorogen near the hole in the cover. These small Petri dishes are placed in a large desiccator containing various test gases. The two photographs in the middle show orientation relative to the hole in the top of the Petri dish. In *a* the desiccator contained water vapour only, and in *b* it contained in addition a partial pressure of 0.0027 mm Hg (0.0036%) of NH<sub>3</sub>. The photographs are taken at an angle from above the Petri dish. The source of NH<sub>3</sub> is above the hole. The lower diagrams make clear how the fruiting bodies orient with respect to the holes. (It has been established by Rorke and Rosenthal<sup>16</sup> that gravity plays a negligible role in the orientation of fruiting bodies.) The NH<sub>3</sub> was produced by placing in the base of the desiccator 800 ml of 0.5 M NaOH and 0.002 NH<sub>4</sub>Cl. We are indebted to Professor Walter Kauzmann for showing us how to estimate the partial pressure of the NH<sub>3</sub> above this solution. Knowing the volume of the air space in the desiccator (2,190 ml), the temperature (23 °C), and using the Henry's law solubility coefficient derived from the literature<sup>17</sup> for dilute aqueous solutions of NH<sub>3</sub>, it is possible to calculate the partial pressure of NH<sub>3</sub> in the air space. (The same method was used for estimating the partial pressures in the experiments on the effect of NH<sub>3</sub> on the rate of cell movement.)

Table 1 The effect of different volatile substances on the orientation of the rising sorogens of *D. discoideum*

	Orientation of fruiting bodies		
	Towards hole	Ignoring hole	Away from hole
Water vapour alone	22	12	0
5% CO <sub>2</sub>	14	9	0
1% ethylene	10	4	0
3–5% ethanol	14	8	0
5–15% ethane	17	7	0
0.0036% NH <sub>3</sub>	2	5	29

These experiments were done in a small Petri dish placed in a large desiccator containing the gases listed above, as described in the text and in the legend to Fig. 1.



a significant increase in the rate of movement of the aggregation streams if the air space above the  $\text{NH}_4\text{Cl}$ - $\text{NaOH}$  solution had a partial pressure of  $\text{NH}_3$  between 0.0025 and 0.0045 mm Hg (Table 2). If the partial pressure exceeded 0.0045 mm Hg the streams broke up and all evidence of cyclic AMP pulses ceased. Pressures of  $\text{NH}_3$  below 0.0025 mm Hg had no apparent effect on the rate of movement of the streams. The speed-up effect was not transient but persisted for an hour or more in the continued presence of  $\text{NH}_3$ .

The variability of the response in each experiment is considerable and may stem from the fact that different branches of an aggregate would move at different speeds even without treatment. Furthermore the cells within a stream move at different rates depending on how far away they are from the aggregation centre; cells at the distal end of a stream may move inwards 2.5 times faster or slower than more proximal ones. Also it is clear when one looks across the thickness of a stream that the cells at the edge move more slowly than those running along the centre. There is obviously a slime sheath that surrounds the streams under our conditions, for cells from outside the stream could join only by attaching to the ends of the stream; they were unable to penetrate the side of the stream. All these properties of amoeba locomotion within streams, including the fact that the cells near the stream sides can obtain traction from the slime sheath, are consistent with the model of Odell and Bonner<sup>10</sup> for the locomotion of migrating slugs. The model requires that all of the amoebae secrete some chemical factor that acts to speed up each amoeba's rate of movement.

In some cases it was possible to measure the rate of individual amoebae that were moving towards a stream, and while the effect of  $\text{NH}_3$  varied in each experiment, the averaged results showed no net change in their rate of movement before and after the addition of  $\text{NH}_3$  (Table 2). Relatively high partial pressures of  $\text{NH}_3$  (0.0045 mm Hg) did increase the frequency of the formation of pseudopods and Samuel<sup>11</sup> showed that this correlated directly with the speed of movement of slime mould amoebae. From this one can only conclude that the effect of  $\text{NH}_3$  on the rate of movement of separate cells (and why it differs from cells in streams) is a matter in need of further investigation.

Since it may easily be shown that, under our conditions, the non-nutrient agar over the  $\text{NH}_3$ -generating solution will become considerably more alkaline in a matter of minutes ( $p\text{H}$  5.5 to 7.0 in less than 5 min), a series of experiments was run to see if  $p\text{H}$  changes alone caused the increase in stream speed. Aggregates were allowed to form on squares of dialysis membranes which were initially placed on agar containing 0.02 M Sorensen's buffer adjusted to a  $p\text{H}$  of approximately 6.0, and after measuring the speed of the cells in a stream the squares were transferred to a similar agar adjusted to a  $p\text{H}$  of approximately 8.0. The speed of the cells did not change significantly. This was also true if the cellophane squares were shifted from the  $p\text{H}$  8.0 agar to the  $p\text{H}$  6.0 agar, thus demonstrating that it is the  $\text{NH}_3$  and not the external hydrogen ion concentration that is responsible for the changes in the speed reported above.

We did some experiments in the middle of the range of effective  $\text{NH}_3$  partial pressures for speeding up aggregation streams (pp $\text{NH}_3$  of 0.0028 mm Hg) and tested for both speed changes and changes in the frequency of the pulses emanating from the aggregation centre before and after treatment, which can easily be counted on the time-lapse video. In three experiments the streams moved from 1.4 to 1.7 times faster, but the average period between pulses either stayed about the same (4.3 to 4.5 min) or increased slightly (4.3 to 6.4 min). This shows that the increase in stream speed cannot be accounted for on the basis of an increase in the frequency of the pulses.

It is known that  $\text{NH}_3$  has other effects on the development of cellular slime moulds. Feit<sup>8</sup> and Lonski<sup>9</sup> showed that it inhibits aggregation centre formation, and later Schindler and Sussman<sup>12</sup>, Thadani *et al.*<sup>13</sup> and Kay<sup>14</sup> provided evidence that it inhibits the production or the effects of cyclic AMP. Schindler and Sussman<sup>13</sup> also demonstrated that the presence of  $\text{NH}_3$

**Table 2** Effect of  $\text{NH}_3$  on the rate of movement of amoebae

	Partial pressure of added $\text{NH}_3$ in mm Hg	Change in speed		No. of cases
		Mean $\pm$ s.d.	Range	
Amoebae in streams	0.0025-0.0045	2.10 times $\pm$ 1.18	1.0 times-6.0 times	23
Isolated amoebae	0.003	1.04 times $\pm$ 0.50	0.5 times-3.0 times	29

Stream speed was measured by placing an acetate sheet over the television screen and marking the outline of the cells, or the streams, at given time intervals (3 min for individual cells; 10 min for streams). It was found that the magnitude of rate increase was variable and did not correlate well with concentration of  $\text{NH}_3$ . The measurements were taken either at the very end of a stream, or on the fastest chains of cells within a segment of a stream, care being taken in each case to measure the same region before and after the addition of  $\text{NH}_3$ . The change in speed is the speed after treatment divided by the speed before treatment.

encourages the migration stage. Finally, Varnum and Soll<sup>15</sup> showed that a constant concentration of cyclic AMP slows the rate of movement of aggregation-competent amoebae, the reverse of the effect of  $\text{NH}_3$  reported here. It is evident that  $\text{NH}_3$  plays a number of important roles in the control of development of the cellular slime moulds. Furthermore one might be able to explain some of the interesting results of past experiments. For instance, as mentioned earlier, slugs will migrate towards acid regions<sup>5,6</sup>. This could be because there will be more free  $\text{NH}_3$  on their basic side, and thus  $\text{NH}_3$  will repel them towards the acid side.

We repeated the main experiments reported here with *Polysphondylium violaceum* in a pilot study and found that while there were differences in detail, *P. violaceum* was repelled by an atmosphere containing 0.03 mm Hg of  $\text{NH}_3$ , and that  $\text{NH}_3$  also increased the rate of movement of the cells in its aggregation streams. This is consistent with the results of our previous experiment in which it was shown that the repellent gas is not species specific<sup>1</sup>.

In conclusion, it is clear that low, physiological concentrations of  $\text{NH}_3$  orient rising fruiting bodies and also increase the speed of cells in a stream. This supports the idea that  $\text{NH}_3$  is the repellent gas responsible for orientation and that it does so by locally increasing the rate of cell movement in cell masses. Thus  $\text{NH}_3$  fits all the criteria needed for the substance postulated by Odell and Bonner<sup>10</sup> that causes cells to move more rapidly in the central axis of a migrating slug.

While this work was in progress we heard from Dr Ira Feit of Franklin and Marshall College that he also has evidence (unpublished) that rising sorogens of *D. discoideum* are repelled by  $\text{NH}_3$ , which is an independent confirmation of the result reported here.

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# The possible role of ammonia in phototaxis of migrating slugs of *Dictyostelium discoideum*

(slime molds/gas orientation/chemotaxis)

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Contributed by J. T. Bonner, February 8, 1988

**ABSTRACT** Previously we showed that the rising cell masses of cellular slime molds orient away from high concentrations of ammonia gas, presumably by speeding up the cells on one side. Here we show that in the same way  $\text{NH}_3$  could also be involved in the highly sensitive phototaxis found in the migrating slugs of *Dictyostelium discoideum*. We have evidence that light increases their speed of migration and their production of  $\text{NH}_3$ . Since unilateral light is concentrated on the distal side of a cell mass by the "lens effect," this leads to the obvious hypothesis that the light stimulates the local production of  $\text{NH}_3$ , which, in turn, stimulates the cells in the illuminated region to move faster.

It has been known for a long time that a gas repels the rising (and migrating) cell masses of the cellular slime molds (1). Recently, we and others (2, 3) have been able to show that the gas involved is  $\text{NH}_3$ . We also gave evidence that  $\text{NH}_3$ , in the range of concentrations that orients cell masses, increases the rate of movement of cells in aggregating streams. This combination of facts produced the proposal that  $\text{NH}_3$  acts as a repellent by speeding up the cells on the side of a cell mass that has the highest concentration of  $\text{NH}_3$ , thereby producing orientation away from the gas.

One of the questions that arose was whether or not the strong phototaxis exhibited by cellular slime molds might be mediated by  $\text{NH}_3$ . It is well known that, as in the mold *Phycomyces*, where it was first discovered by Buder (4), slime mold slugs concentrate or focus the light on the distal side of the cell mass, the so-called "lens effect" (5, 6). As Buder did for *Phycomyces*, Francis (7) concentrated a minute spot of light from above on one side, near the tip (the sensitive region) of the migrating slug of *Dictyostelium discoideum* and showed that the illuminated cells on that side move faster and the cell mass curved away from the spot of light. If light and  $\text{NH}_3$  speed up cells within a slug, is it possible that a concentration of light causes a local increase in the production of  $\text{NH}_3$ , which, in turn, causes the differential speedup of the cells on the distal side? Here we present evidence that supports such a hypothesis.

## MATERIALS AND METHODS

The experiments were done with *D. discoideum* NC-4 grown on *Escherichia coli* B/r. To prepare the slugs for measuring the effects of  $\text{NH}_3$  on the speed of slugs, amoebae were grown on nutrient agar plates (buffered 1% peptone/1% dextrose/2% agar), washed by centrifugation, and placed in small, concentrated drops on 2%, nonnutrient agar. After the slugs migrated away from the drop, individuals were isolated on a circle of agar with a cork borer and placed in a corresponding hole on a fresh 2% agar Petri dish. This was then inverted

over a crystallizing dish (Pyrex 40 × 80 mm) containing 5 ml of 1 mM  $\text{NH}_4\text{Cl}$  and 5 ml of 1.0 M NaOH. The partial pressure produced in the chamber was 0.0065 mmHg. The rates of movement of the slug were measured from video tape taken through a 50-mm lens attached through a microscope to a Panasonic video camera (WV-1850) with time-lapse (AG-6010). The experiments were run at 17°C.

The phototaxis experiments using unilateral light were done in a blackened wooden box (50 × 50 × 50 cm) in which a frosted Plexiglas cylindrical rod (2 cm in diameter) entered vertically down one side of the box. A 15-W incandescent bulb was placed over the rod, but separated from it by a flask containing a 5% solution of  $\text{CuSO}_4$  to cool the light by filtering out the wavelengths above 650 nm while preserving those important for phototaxis. Covered Pyrex crystallizing dishes (90 × 50 mm) were fitted with a nylon screen rack that came up above the  $\text{NH}_3$ -generating solution. Right side up on the rack a small, open plastic Petri dish bottom (60 × 15 mm, Falcon no. 1007) was placed that contained 2% nonnutrient agar and a central plug of migrating slugs. The plugs were punched out of a nutrient agar growth plate at the end of the aggregation stage. The crystallizing dishes were placed 10–15 cm from the illuminated bar on shelves within the light-tight box (kept at 17°C).

To see if light affects the amount of  $\text{NH}_3$  given off by migrating slugs, the slugs were prepared by washing the amoebae off plates before aggregation (as described above) and the cells in the centrifuge pellet were diluted to  $4 \times 10^7$  cells per ml. One milliliter of such a suspension was spread on a 2% nonnutrient agar Petri plate (100 × 15 mm), allowing the excess liquid to evaporate. Slugs developed within 16 hr in the dark at 17°C. For each experiment, eight Petri plates with the slugs were placed upside down and a small Petri dish bottom containing 8 ml of distilled water was inserted inside each Petri dish so that its edges were flush against the agar ceiling containing the migrating slugs. Four dishes were then put in the dark box and four were put in a box with a slit illuminated by unilateral light from a fiber optic lamp, all at 17°C. The water was then changed and the plates were put under the reciprocal conditions for another 2-hr period. To measure the  $\text{NH}_3$  that had accumulated, the four dishes of water from each condition were pooled and tested for  $\text{NH}_3$  by using an Orion  $\text{NH}_3$  electrode and an Orion expandable ion analyzer, model EA 940. The method of collecting and measuring the  $\text{NH}_3$  is that of Ira Feit, who kindly gave us advice and the benefit of his experience.

## RESULTS

**Effect of  $\text{NH}_3$  on Slug Speed.** At first we had great difficulty with these experiments for we used plates with many slugs on them and there was no difference in their speed before and after adding  $\text{NH}_3$ . We thought that this might be because all of the slugs on the plate were giving off  $\text{NH}_3$  that was saturating the environment before any further  $\text{NH}_3$  was added. If only one or two slugs were placed in the dish, then



we were able to obtain differences in the speed before and after the addition of the  $\text{NH}_3$ -generating solution. In 16 experiments where each slug was measured for 40 min, the mean speed ( $\pm$ SEM) over water was  $1.31 \pm 0.02$  mm/hr ( $n = 16$ ) and for the same slugs after the addition of  $\text{NH}_3$  it was  $1.45 \pm 0.02$  mm/hr ( $n = 16$ ). By using a paired  $t$  test (one-tailed), the difference is significant at the level of  $P < 0.05$ .

**Slug Movement in the Absence of  $\text{NH}_3$ .** We also performed some experiments in which the  $\text{NH}_3$  was removed following the enzymatic method used by Schindler and Sussman (8). When a slug was placed on a Nuclepore filter (pore size = 5  $\mu\text{m}$ ) over a filter pad soaked with a mixture of 25 mM ketoglutarate/25 mM NADH/60 activity units of glutamine dehydrogenase/20 mM Na/K phosphate buffer, pH 7.3, in a small, well-closed Petri dish (50  $\times$  9 mm; Falcon no. 1006), all forward movement of the slug ceased. This was not the case when any one of the three components of the mixture was tested separately in buffer, indicating that the key factor in preventing locomotion was probably the absence of  $\text{NH}_3$ .

**Orientation to Unilateral Light in an Atmosphere of  $\text{NH}_3$ .** One way to test if  $\text{NH}_3$  might be involved in phototaxis by locally speeding up cells in the slug is to saturate the atmosphere with a nontoxic level of  $\text{NH}_3$  around slugs that are illuminated from one side to see if the excess  $\text{NH}_3$  cancels out the normal phototactic response. This experiment works remarkably well and has been repeated many times. As can be seen in Fig. 1, if two plates of slugs in identical containers with the same source of unilateral light are compared, the slugs over water show normal phototaxis, whereas the slugs over  $\text{NH}_3$  show little or no response to the light.

Another way in which the absence of phototaxis in an atmosphere of  $\text{NH}_3$  can be shown is by having slugs crawling on the surface of agar in a Petri dish inverted over a crystallizing dish (80  $\times$  40 mm) containing an  $\text{NH}_3$ -generating solution (again involving a range of partial pressures from 0.0065 to 0.026 mmHg of  $\text{NH}_3$ ). The slugs are not affected by lateral light or light from above (as they are in controls), which makes them hug the agar. Instead, the normally inconsequential effect of gravity takes over and a very high percentage of the slugs points straight downward, often continuing to migrate so that they become suspended on a

thread of slime sheath, ultimately fruiting in midair (Fig. 2). If the plates are right side up in an atmosphere of  $\text{NH}_3$ , no such effect is seen. Nor do they orient in the fashion shown in Fig. 2 if they are inverted over water in the dark.

**Effect of Light on the Emission of  $\text{NH}_3$ .** Again we tried numerous ways to do this experiment and finally adopted a method devised by Ira Feit (personal communication), where nonnutrient agar plates containing a heavy concentration of washed amoebae are inverted over an open Petri dish containing distilled water when the cells have reached the migration stage. The water is collected after a 2-hr interval and measured for  $\text{NH}_3$  with the  $\text{NH}_3$  electrode. Such plates were put in a box with a slit and given unidirectional light for 2 hr and then placed in the dark in a light tight box for 2 hr, or the sequence was reversed. In 20 such experiments the mean  $\text{NH}_3$  concentration was 15  $\mu\text{M}$  in the light and 11  $\mu\text{M}$  in the dark. Since the measurements were made in millivolts, the means  $\pm$  SEM are  $-29.8 \pm 3.7$  mV in the light and  $-22.2 \pm 3.5$  mV in the dark. In a paired  $t$  test (one-tailed) the difference is significant at the level of  $P < 0.005$ . It is unfortunate that it is not possible, because of the difficulty in obtaining accurate  $\text{NH}_3$  measurements at such low concentrations, to do a dose-response curve to see how much the amount of  $\text{NH}_3$  given off by the slugs varies with light intensity.

## DISCUSSION

We have given evidence to support the idea that not only do gradients of  $\text{NH}_3$  orient cell masses of slime molds but also that this phenomenon could be involved in positive phototaxis: the light is concentrated on the distal side by the lens effect, more  $\text{NH}_3$  is presumably generated in the illuminated region, and this increase stimulates the cells in that region to move more rapidly, thereby causing orientation toward light. The evidence that we have to support such a hypothesis is that (i)  $\text{NH}_3$  causes cells in the slug to move more rapidly and a removal of  $\text{NH}_3$  causes them to stop moving, (ii) light increases the amount of  $\text{NH}_3$  given off by the cells, and (iii) an excess of added  $\text{NH}_3$  inhibits phototaxis but not migration.

Such a hypothesis fits in well with the experiments of Kitami (9), who showed that slugs of *D. discoideum* migrating against centrifugal forces moved more rapidly toward light than in the dark. As he points out, this is in keeping with the earlier observations of Poff and Loomis (10), who showed that slugs moving phototactically increase their rate of

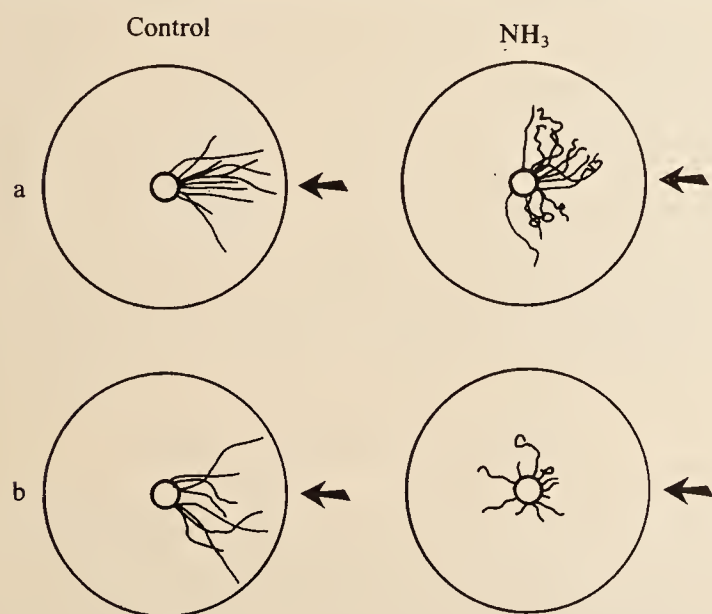


FIG. 1. Effect of  $\text{NH}_3$  on phototaxis. Two experiments showing slime tracks of slugs that have migrated out from a central plug of agar in a Petri dish. The arrows show the direction of the light source. Note that in an atmosphere of  $\text{NH}_3$ , the slugs become disoriented and fail to move toward the light. This is partially true in *a*, where the partial pressure of the  $\text{NH}_3$  is 0.005 mmHg, and completely so in *b*, where the partial pressure is 0.008 mmHg.

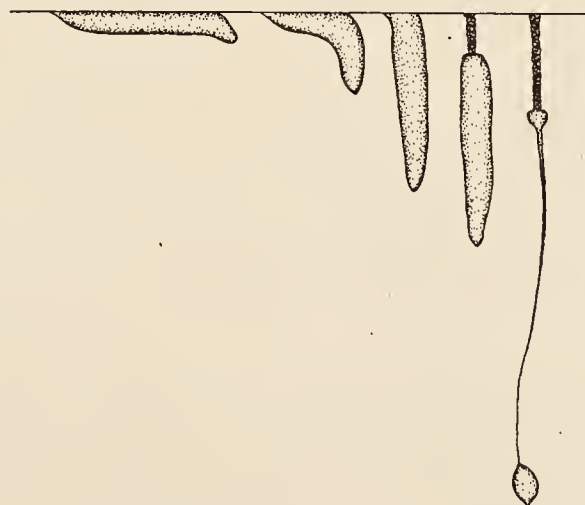


FIG. 2. Semidiagrammatic drawing in a time sequence showing (from left to right) that if slugs are migrating on the ceiling of a dish containing  $\text{NH}_3$  vapor, gravity appears to take over and the slugs point straight downward. Note the migration continues off the surface of the agar so that the fruiting body is suspended on a thread of slime sheath. (Also note the curious shape of the basal disc under these circumstances.)



movement with the intensity of illumination. As one can see from their data, a  $10\times$  increase in illumination produces a  $1.3\times$  increase in speed. However, it should be pointed out that Smith *et al.* (11) find no difference in speed under similar conditions. Also a number of workers have tried to see if general, undirected illumination affects the rate of speed, but no difference could be observed between light and dark (5, 12).

One other fact in the literature fits in with the  $\text{NH}_3$ -speed hypothesis, although this point is far more speculative and fanciful. It has been known for a long time that larger slugs move more rapidly than smaller ones (13–15). Could this be because a greater number of cells will produce a larger concentration of  $\text{NH}_3$ ? This would also account for the well-known fact that very small slugs do not migrate at all but fruit immediately. [This also fits in with Schindler and Sussman's (8) evidence that  $\text{NH}_3$  favors migration over fruiting.]

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# Ammonia and thermotaxis: Further evidence for a central role of ammonia in the directed cell mass movements of *Dictyostelium discoideum*

(slime molds/taxes/kineses/pH)

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**ABSTRACT** Evidence is presented to support the hypothesis that, in addition to its possible role in mediating chemo- and phototaxis, ammonia ( $\text{NH}_3$ ) is also the key substance responsible for directing thermotaxis of the migrating slugs of *Dictyostelium discoideum*.  $\text{NH}_3$  is produced by the cells of the slug and we show that high and low concentrations of  $\text{NH}_3$  decrease the speed of the amoebae while intermediate concentrations increase their speed.  $\text{NH}_3$  production by amoebae is affected by temperature: the greater the temperature, the more  $\text{NH}_3$  is produced. From these facts we speculate that both the positive and the negative thermotaxis found in slugs can be explained by temperature gradients stimulating regional differences in  $\text{NH}_3$  production, and depending upon the temperature, the amount of  $\text{NH}_3$  will either be in the range that stimulates or inhibits the rate of movement. If this explanation is correct, then minute localized differences in the production of  $\text{NH}_3$  and their differential effect on cell speed could account for all the directed movements of the cell masses of these slime molds.

In previous studies we have shown that ammonia ( $\text{NH}_3$ ) plays a key role in orienting cell masses by speeding up the amoebae on one side of a migrating or culminating cell mass, thereby causing a turning by means of the differential forward motion of the internal amoebae. In this way one may account for the orientation of cell masses away from one another (negative chemotaxis) and for positive phototaxis where more light causes an increase in the local production of  $\text{NH}_3$  which in turn speeds up the cells in that region (1–4). Here we present evidence that thermotaxis might also be mediated in the same way. During the course of this investigation we examined in detail: (i) the production of  $\text{NH}_3$  at various stages of the life cycle of *Dictyostelium discoideum*; (ii) the effect of various concentrations of  $\text{NH}_3$  on the rate of movement of isolated amoebae and of migrating slugs; (iii) how the  $\text{NH}_3$  produced by an individual slug can affect the speed of its own movement; and (iv) how temperature affects  $\text{NH}_3$  production in slugs. All these studies contribute to our understanding of the way  $\text{NH}_3$  might play a central role in all the directed morphogenetic movements of the cellular slime molds.

## MATERIALS AND METHODS

These experiments were done with *Dictyostelium discoideum* NC-4 grown on *Escherichia coli* B/r.

**Life Cycle Experiments.** Amoebae were grown on bacteria in Petri plates containing buffered 2% (wt/vol) agar with 1% peptone and 1% dextrose. The  $\text{NH}_3$  given off was collected by placing bottoms of plates covered with a lawn of amoebae

upside down over the lid containing a smaller Petri plate (68 × 9 mm deep) with 8 ml of distilled water. The water was changed at 2-hr intervals throughout development. Each experiment involved three plates and was repeated four to nine times (at 23°C under ceiling fluorescent lights). The  $\text{NH}_3$  content of the pooled water from each interval was measured with an  $\text{NH}_3$  electrode (Orion expandable ion analyzer, EA 940).

**Effect of  $\text{NH}_3$  on Rate of Movement of Amoebae.** Petri plates, each containing a drop of amoebae (from a suspension of  $2 \times 10^6$  cells per ml) on 2% agar, were inverted over crystallizing dishes (Pyrex, 40 × 80 mm) containing first 10 ml of distilled water and then 5 ml of various concentrations of  $\text{NH}_4\text{Cl}$  combined with 5 ml of 1.0 M NaOH. The movements of the amoebae at 17°C were recorded on a video tape using a Panasonic video camera (WV-1850) with time lapse (AG-6010) feature mounted on a microscope with a 6.3-mm lens. Their speed was calculated from tracings on the screen for 30-min intervals.

**Effect of  $\text{NH}_3$  on Rate of Movement of Slugs.** Sets of three Petri plates, each containing a single slug, were inverted over various concentrations of  $\text{NH}_3$ -generating solutions (and  $\text{H}_2\text{O}$  controls). The experiments were done for 1 hr at 17°C in the dark, and the distance traversed by the slugs was determined by measuring the track lengths.

**Effects of Decreasing Rather Than Increasing the Internal pH of the Cells.** We examined the effects of  $\text{CO}_2$  on slug speed. The procedure was the same as with  $\text{NH}_3$ . A gas of 5%  $\text{CO}_2$ /95% air (from a cylinder) was bubbled through 50 ml of distilled water in a crystallizing dish for 5 min and plates with single slugs were inverted over the dishes. These experiments were also done at 17°C. Parallel experiments on a drop of separate amoebae ( $2 \times 10^6$  cells per ml) were recorded and measured on the video screen.

**Thick-Thin Agar Experiments.** These experiments were done a number of ways, all giving the same result, but the most successful method was to submerge a small plastic Petri plate lid (50 × 3 mm, Falcon, 1007) right side up in a larger Petri plate (100 × 15 mm) and to pour in 2% agar so the submerged lid was just covered with agar. The rates of the slug migration at 17°C were measured using the time-lapse video camera.

**Temperature Gradient Experiments.** A temperature gradient was devised by placing a covered 40-W G.E. Showcase light bulb controlled by a variable resistor (Variac) onto an aluminum block (30 × 12 × 2 cm) placed into a cold incubator (12°C). The temperature gradients were measured by two thermocouples. Three Petri plates (100 × 15 mm), each containing a drop of washed amoebae that had been allowed to develop at 17°C in the dark on 2% (non-nutrient) agar, were placed across the aluminum block. The  $\text{NH}_3$ -generating solution was added to a small Petri dish bottom at the side of the plate (see Fig. 5).

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**Effects of Temperature on  $\text{NH}_3$  Emission.** Amoebae were grown at specified temperatures to the end of the vegetative stage, washed by centrifugation, plated out on 2% agar at a concentration of  $4 \times 10^7$  cells per plate ( $100 \times 15$  mm), and allowed to develop into slugs in the dark at specified temperatures. Plates of slugs were then inverted over their lids containing smaller Petri dishes ( $68 \times 9$  mm) with 8 ml of distilled water, and three such plates were put into each of the experimental temperatures in the dark for 4 hr. The water aliquots were pooled and tested for  $\text{NH}_3$  content by using the  $\text{NH}_3$  electrode.

## RESULTS

**Production of  $\text{NH}_3$  During the Course of Development.** Cellular slime molds produce  $\text{NH}_3$  during the developmental stages (5–10). Here we examined the time course of  $\text{NH}_3$  production from the vegetative stage to midculmination, and as can be seen from Fig. 1, there is a steady increase in  $\text{NH}_3$  and the multicellular stages give off more  $\text{NH}_3$  than the vegetative stage.

**Effects of  $\text{NH}_3$  on the Rate of Movement.** The speed of both individual amoebae and migrating slugs was measured under various partial pressures of  $\text{NH}_3$  and then plotted as percent differences from the control without the  $\text{NH}_3$ . Every effort was made to follow the same amoebae before and after the addition of  $\text{NH}_3$ . The results from experimental slugs were compared with results from a separate set of control slugs obtained on the same day.

As can be seen in Figs. 2 and 3, the scatter of the data is considerable, yet it is clear that there is a central peak where  $\text{NH}_3$  speeds up both cells and slugs, and at  $\text{NH}_3$  concentrations above the peak the speed is inhibited. It is interesting to note that the range of  $\text{NH}_3$  concentrations where speed is increased is greater for slugs (0.2–0.9 mM) than for amoebae (0.04–0.2 mM). The reason for this is not obvious, although it could simply be that the slime sheath of the slug is less permeable to  $\text{NH}_3$  than the membrane of a single cell.

**Effects of  $\text{CO}_2$  on Movement of Amoebae and Slugs.** On the assumption that the increased speed is in some way related

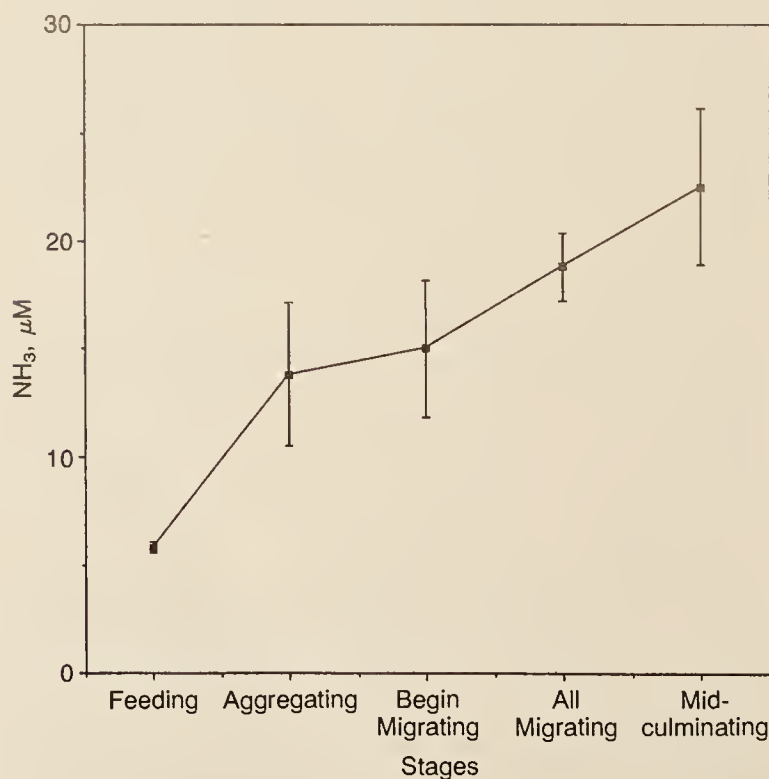


FIG. 1. Amount of  $\text{NH}_3$  given off by *D. discoideum* at various stages of development. The points are an average of four to nine experiments, each of which involves three Petri dishes of developing slime molds, and the bars are the SEM for each point. The amount of  $\text{NH}_3$  collected (ordinate) is proportional to the rate of  $\text{NH}_3$  production per amoeba per unit time.

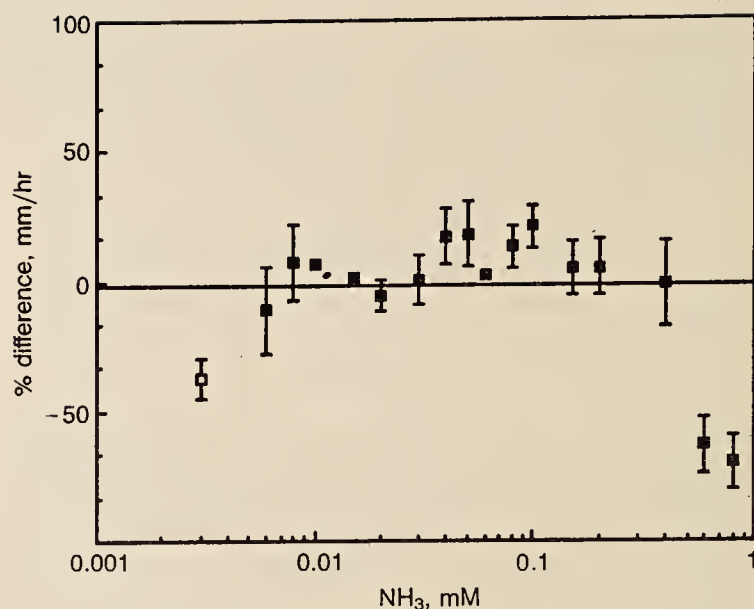


FIG. 2. Percent differences of the rates of movement of vegetative amoebae under various concentrations of  $\text{NH}_3$  before and after the addition of the  $\text{NH}_3$ . The solid squares are the average of three or four experiments, each involving 6–10 amoebae, which were measured for 30 min before and after the addition of  $\text{NH}_3$ . The percent difference between the control and the experimental is shown for various concentrations of  $\text{NH}_3$ . The bars show the SEM for each point. [To convert to partial pressure divide the concentration (mM) by 190 to give the partial pressure (mmHg).] A similar experiment was done 12 times with 5%  $\text{CO}_2$ /95% air (open square).

to the  $\text{NH}_3$  causing an increase in pH inside the cells, we tested the speed of amoebae and slugs in an atmosphere of 5%  $\text{CO}_2$ /95% air, which should have the opposite effect. As can be seen in Figs. 2 and 3,  $\text{CO}_2$ , which would be expected to lower the pH of the cells, generally causes a reduction in the rate of movement.

**Slug Speed on Agar Thickness.** Another way to see the effects of  $\text{NH}_3$  on the rate of movement in slugs is to have them migrate toward a light from thick agar (2.3–3.3 mm deep) to shallow agar (0.6–0.9 mm deep). The speed is significantly faster over the thin agar. [The mean speed for eight slugs was  $1.51 \pm 0.13$  mm/hr (SEM) and for the same slugs  $1.19 \pm 0.08$  mm/hr (SEM) over thick agar.  $P < 0.05$ .] This is well illustrated in the best case of several runs where

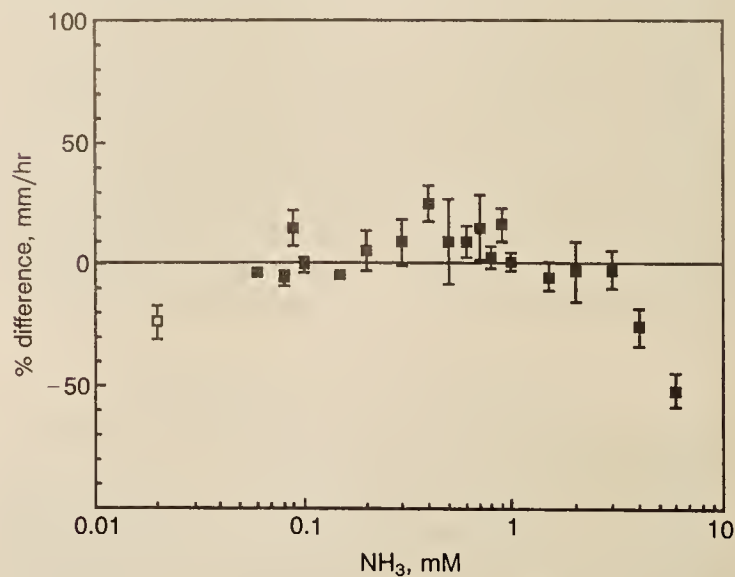


FIG. 3. Percent differences of the speed of migrating slugs over various concentrations of  $\text{NH}_3$  versus the controls where no  $\text{NH}_3$  was added. Each solid square is the average of three or four experiments ( $\pm$  SEM). On each day of an experiment, sets of three Petri plates, each containing one slug, were subjected to various concentrations of  $\text{NH}_3$  and each of these sets was compared to one set of three control plates with no added  $\text{NH}_3$ . A similar experiment was done nine times with 5%  $\text{CO}_2$ /95% air (open square).

we have a continuous record of a single slug that went from thick to thin agar (Fig. 4A). Clearly by the time at least half of the slug is over the thin agar the speed increases.

It is obvious we cannot prove that this effect is due to  $\text{NH}_3$  and not some other diffusible substance.  $\text{NH}_3$  is, however, the most likely candidate for a very interesting reason. It was difficult to lead the slug over the thin agar with a weak directional illumination. For each success there were many failures, where, as soon as the slug tip reached the edge of the thin agar over the submerged plastic platform, the slug would shy away by turning (Fig. 4B). Since a local high concentration of  $\text{NH}_3$  has been observed to cause such repulsion of cell masses (2, 3), one can reasonably postulate that the  $\text{NH}_3$  diffusing into the thin agar reaches a higher local concentration than in the thick and, consequently, repels.

**$\text{NH}_3$  and Thermotaxis.** The first way we examined the question of whether  $\text{NH}_3$  was involved in thermotaxis was to repeat the kind of experiment we had done for phototaxis (4), that is, to put slugs on a temperature gradient and flood the atmosphere with  $\text{NH}_3$  at various concentrations. This was done for both positive and negative thermotaxis, the latter having been demonstrated by Whitaker and Poff (11). They showed that thermotaxis is positive if the gradient is above the growth and development temperature and negative if it is below. In both cases added  $\text{NH}_3$  disoriented the slugs, although the effect is less striking in negative chemotaxis, probably because our temperature gradient was suboptimal (Fig. 5). Note that as the concentration of  $\text{NH}_3$  increased, the length of the tracks of the slugs decreased, clearly indicating an inhibition of speed by the  $\text{NH}_3$ . This would be expected for a  $\text{NH}_3$  concentration of 6 mM as can be seen in Fig. 3.

**Effect of Temperature on  $\text{NH}_3$  Production.** Next we examined the effect of temperature on the emission of  $\text{NH}_3$  by migrating slugs, and, as can be seen from Fig. 6, the higher the temperature, the more  $\text{NH}_3$  was produced. Furthermore, the emission was affected by the temperature at which the amoebae developed: those cultures that developed at higher temperatures gave off more  $\text{NH}_3$  at any one temperature than those that developed at a cooler temperature (Fig. 6).

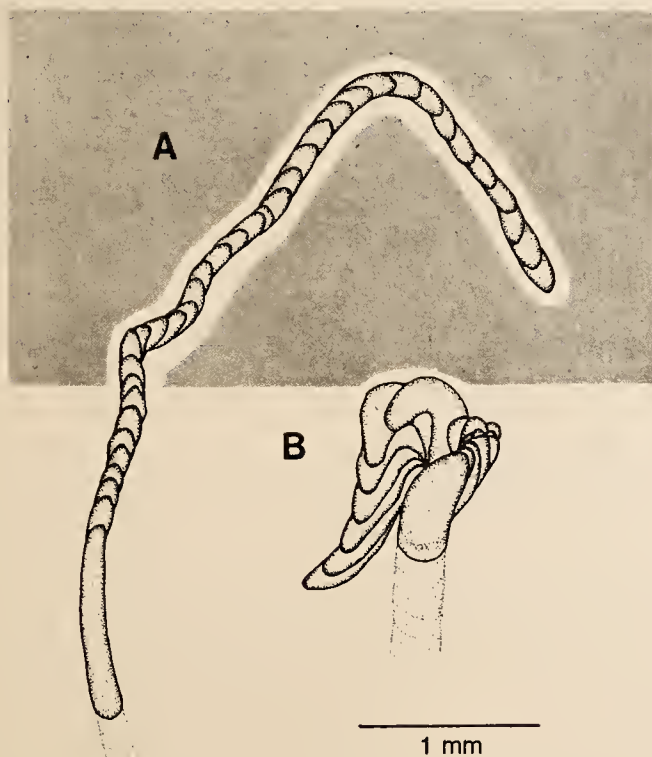


FIG. 4. (A) Tracings from a video screen taken at 10-min intervals of a migrating slug going from thick (white background) to thin (gray background) agar. Note that once the slug is over the thin agar it moves more rapidly. (B) Similar tracings of a slug that has turned upon reaching the edge of the thin agar.

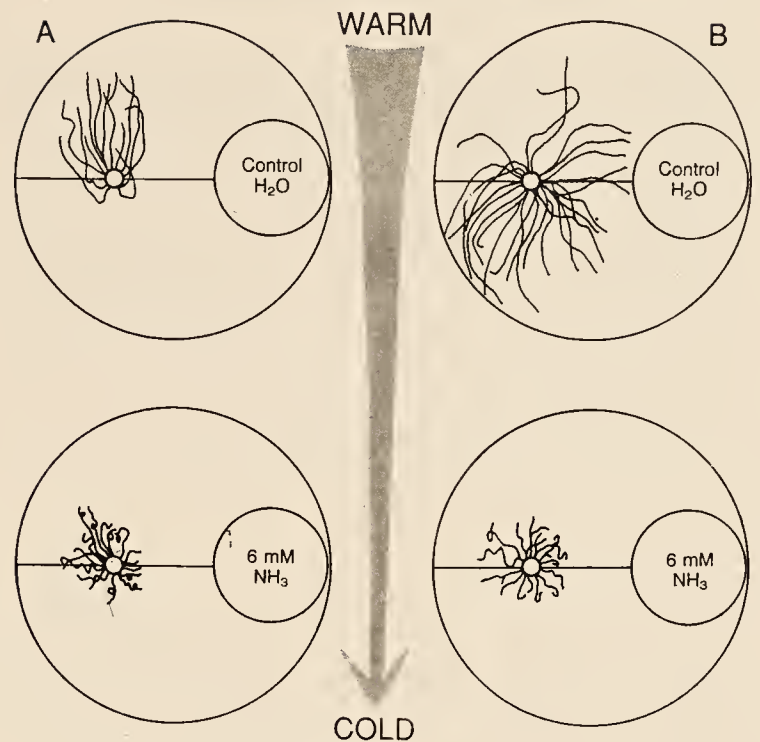


FIG. 5. Effect of adding  $\text{NH}_3$  on the orientation of slugs in a heat gradient. (A) Positive thermotaxis of slugs in a temperature gradient above the temperature at which development occurred. The control (Upper) shows strong orientation toward the warmer side, but the orientation is inhibited by 6 mM  $\text{NH}_3$  (Lower). (B) Negative thermotaxis in a temperature gradient below the development temperature. The slugs in the control show greater orientation toward the colder side than do those of the experimental plate in an atmosphere of  $\text{NH}_3$ .

## DISCUSSION

The simplest model for all these results assumes that  $\text{NH}_3$  directly controls the rates of movements of the amoebae inside a slug; the amoebae are all moving in the same direction toward the anterior tip of the slug due to the internal

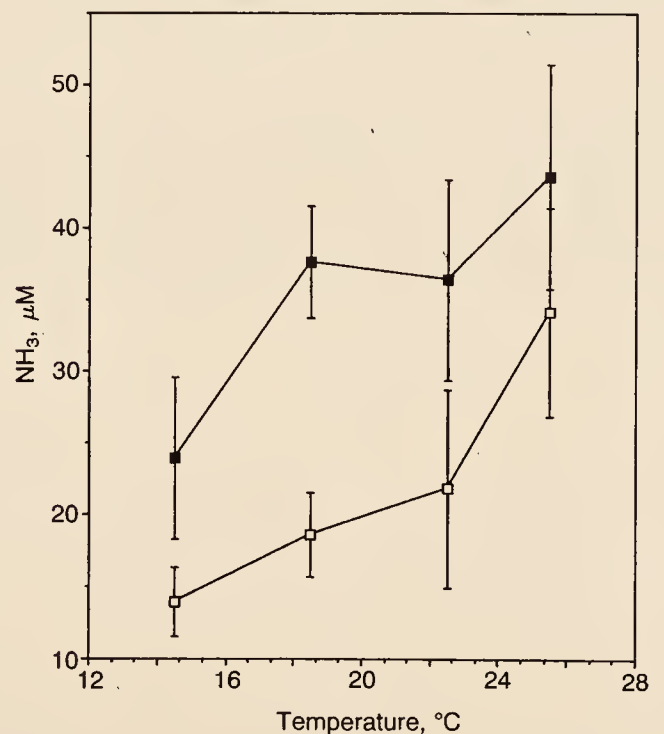


FIG. 6. Effect of temperature on  $\text{NH}_3$  production of migrating slugs. Note that although  $\text{NH}_3$  production consistently increases with temperature, the effect is greatly influenced by the temperature at which the slugs develop. Each point is the mean of four to nine experiments, each consisting of three plates of slugs, and the bars are the SEM for each point. Developmental temperatures: ■, 22–23°C; □, 18–19°C.



antero-posterior pulse gradient of cAMP (12–14). If one removes all the  $\text{NH}_3$  produced by the slug using an enzymatic method, then all movement stops (4). What we have done here is compare the rate of movement of isolated slugs with and without added  $\text{NH}_3$ ; in other words, we are comparing slugs with two levels of  $\text{NH}_3$  and then we find that some addition of  $\text{NH}_3$  causes an increased speed, while the addition of even more  $\text{NH}_3$  results in an inhibition of the rate of cell movement (Figs. 2 and 3). This means that in the earlier work on repulsion of cell masses (2, 15) the concentration of  $\text{NH}_3$  that repelled slugs was in the optimal range for increased cell speed. The same is true for the effect of directional light that is concentrated on the far side of the slug by the “lens effect”; again the light must be concentrated enough to cause the cells to produce the optimum amount of  $\text{NH}_3$  for fast cell movement.

In the present study of thermotaxis there is the further interesting complication that, besides the long-established positive thermotaxis, Whitaker and Poff (11) showed that positive thermotaxis occurs only in temperature gradients spanning temperatures higher than the temperature at which the amoebae developed. Further, if the slugs are subjected to gradients spanning temperatures less than the development temperature, they are negatively thermotactic and move away from the warmer side. (See controls in Fig. 5.)

The model can easily encompass these facts for we have shown that the higher the temperature the more  $\text{NH}_3$  is produced and that the speed of movement of the amoebae is  $\text{NH}_3$  dependent: low  $\text{NH}_3$  stimulates the rate of cell movement (over a restricted range of concentrations) and high  $\text{NH}_3$  inhibits the rate of cell movement. For negative thermotaxis, where the overall production of  $\text{NH}_3$  is relatively low, we postulate that the amount of  $\text{NH}_3$  on the warmer side of the slug is within the  $\text{NH}_3$  concentration range that stimulates cell movement, and for this reason the slug orients away from heat. For temperature gradients above the developmental temperature, the overall production of  $\text{NH}_3$  has been pushed up so high we postulate that the  $\text{NH}_3$  concentration on the warmer side of the slug is in the inhibitory range and, therefore, the amoebae on the far side move faster and orient the slug toward the heat.

This model raises some interesting questions. First, how does  $\text{NH}_3$  stimulate or inhibit cell locomotion? Perhaps locomotion is affected through pH (an idea supported by our  $\text{CO}_2$  results), because increased intracellular pH results in the increased activity of contractile proteins and cell motility [for a review, see Simchowitz and Cragoe (16)]. Furthermore, an

increase in pH stimulates chemotactic movement in leukocytes (16).

Second, how is the production of  $\text{NH}_3$  within the slugs controlled? We do not know whether there is a specific deamination reaction that responds to small changes in temperature and light. Perhaps the most puzzling aspect of the tactic responses in these slime mold slugs is that differences in temperature as small as 4–5/10,000 of a °C across the tip of a slug are sufficient to cause orientation (17, 18). This means that the response system must be exquisitely sensitive to minute differences in temperature and light and that these differences are somehow amplified, through the aegis of  $\text{NH}_3$ , into sizeable differences in cell speed.

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